# DEVELOPMENT OF HEART FUNCTION IN THE SHRIMP *METAPENAEUS ENSIS*

M. Phil. (BIOLOGY)

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by

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## ABSTRACT

During development, physiological structures and functions develop to meet the growing demands of animals. One of the systems which undergoes major changes during development is the cardiovascular system. There have been few studies specifically on the functional development of the heart or circulatory system in invertebrates. The present study aims to provide information on ontogenic changes in heart function and heart innervation, and the response of heart rate to salinity and temperature variation during larval development of the shrimp *Metapenaeus ensis*. Micro-videophotography technique was the main investigation method used in the present study and dil labeling technique was used in the study on heart innervation.

The study on change in heart rate during development showed that heartbeat in *Metapenaeus ensis* was first observed in nauplius VI instar. The heart rate increased dramatically from protozoea I and reached a peak at protozoea III but declined gradually from mysis I onwards. A biphasic relationship was found between heart rate and body weight during development. Heart rate increased at 1.70 power of body weight from nauplius VI to protozoea III and decreased at 0.34 power of body weight from protozoea III to benthic postlarvae. There was a change in heart shape during development which may represent different stages of the development of the circulatory system. Starting from mysis II and III instars, heartbeat of the shrimp would stop for a few milliseconds in response to a great hit applied on the bench during measurement of heart rate. This response suggested that mysis II and III was the suspected transitional stage from a myogenic heart in earlier stages to a neurogenic heart in adult.

The study on effect of salinity variation on heart rate showed that the heart rate of protozoea III, mysis III and planktonic postlarvae reared at 18‰ was higher than those reared at 28‰ while the heart rate of benthic postlarvae was not different at the two salinities. The different responses in different early developmental stages are possibly related to ontogenic changes in salinity tolerance, osmoregulatory ability, iso-osmotic point and control mechanism of cardiac function. Heart rate of larvae and postlarvae increased when shrimp reared at 28‰ were exposed to 18‰, indicating an acute reduction in salinity is stressful to the shrimp in all stages. However, when shrimp reared at 18‰ were exposed to 28‰, heart rate did not change in all stages studied except mysis III where a decrease in heart rate was

observed. This result suggested that elevated heart rates which develop in acclimation to low salinity conditions do not respond readily to an acute increase in salinity.

The study on effect of temperature variation on heart rate showed that the response of heart rate of the shrimp raised at 25°C to acute decrease in ambient temperature (20°C) is the same in larvae and postlarvae in which heart rate decreased with decrease in temperature. However, the response of heart rate to acute increase in ambient temperature in larvae (30°C) resembled that of crustaceans measured outside the temperature capacity range in which heart rate becomes irregular or did not change with temperature. While the response of heart rate in postlarvae to the same acute increase in ambient temperature resembled that of the crustaceans measured within the temperature capacity range in which heart rate increased with temperature. The different responses in different early developmental stages are possibly related to the life cycle of *Metapenaeus ensis* and ontogenic changes in temperature capacity range, optimum temperature, and control of cardiac function.

The study on heart innervation during development demonstrated that DiI labeling is applicable as a neural tracer in crustaceans as it does in vertebrates. Though no direct pathway from ventral nerve cord to the heart was found, a pair of nerve bundle was found to originate from thoracic ganglion 3 and 4, and went close to the position of the heart. This pair of nerve bundle were possibly involved in the control of heart function.

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# CHAPTER 1

# INTRODUCTION

During development, physiological structures and functions develop to meet the growing demands of animals. One of the systems which undergoes major changes during development is the cardiovascular system. A review by McMahon *et al.* (in press) showed that there have been few studies specifically on the functional development of the heart or circulatory system in invertebrates. Physiological studies on early developmental stages are necessary for understanding the physiological adaptations of these stages to environmental stress, particularly since these may involve responses quite different from those of the adult. This thesis presents information on ontogenic changes in heart function and heart innervation, and the response of heart rate to temperature and salinity during development of the shrimp *Metapenaeus ensis*.

The specific objectives are:

(1) to examine the changes in heart rate during development of *Metapenaeus ensis* and to investigate the relationship between heart rate and body weight,

(2) to examine the effect of salinity on heart rate during development,

(3) to examine the effect of temperature on heart rate during development, and

(4) to examine the heart innervation during development.

Results of the changes in heart rate during development and its relationship with body weight should allow better understanding of ontogenic changes in heart function associated with development. Results on the effect of salinity and temperature on heart rate during development would provide valuable information for the comparison of physiological responses to environmental stress between different developmental stages. Information from the heart innervation during development would establish a link between cardiac innervation and changes in cardiac function during development. Such information is essential for further studies on the control mechanisms of cardiac function during development.

This thesis consists of 7 chapters. Chapter 1 is a brief introduction of the study. Chapter 2 is a review of the relevant literature on crustacean circulatory system and on the effects of temperature and salinity on cardiac function. The aim is to present an overall picture of the important concepts and information in this field of study. The changes in heart rate during development of *Metapenaeus ensis* are reported in Chapter 3. The effects of temperature and salinity variation on the heart rate during development are presented in Chapter 4 and 5, respectively. A preliminary study of the heart innervation during development is reported in Chapter 6. Chapter 7 is the conclusions of the general findings of this study.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 General Review on Crustacean Circulatory System

### 2.1.1 General anatomy and function

The crustacean circulatory system is an open system in which the blood or hemolymph flows freely throughout the hemocoelic cavity. Hemolymph baths the tissues and returns to the heart via open hemocoelic sinuses. Maynard's (1960a) review includes a comprehensive account on the range of complexity found in the open circulatory systems of crustaceans. Open circulatory systems of crustaceans range from extremely simple, as in some copepods with only a single tubular contractile vessel to the very complex system of the higher decapods. The compact, triangular heart has three pairs of ostia (two dorsal and one ventral) and is contained in a pericardium just in front of the posterior dorsal edge of the carapace.

The arterial systems among the decapods are quite similar (McLaughlin, 1983, review). The hemolymph leaves the heart through five major set of arteries: a single anterior aorta, a pair of anterior lateral arteries, a pair of hepatic arteries, a single posterior aorta and a single sternal artery (Fig. 2.1). The anterior aorta

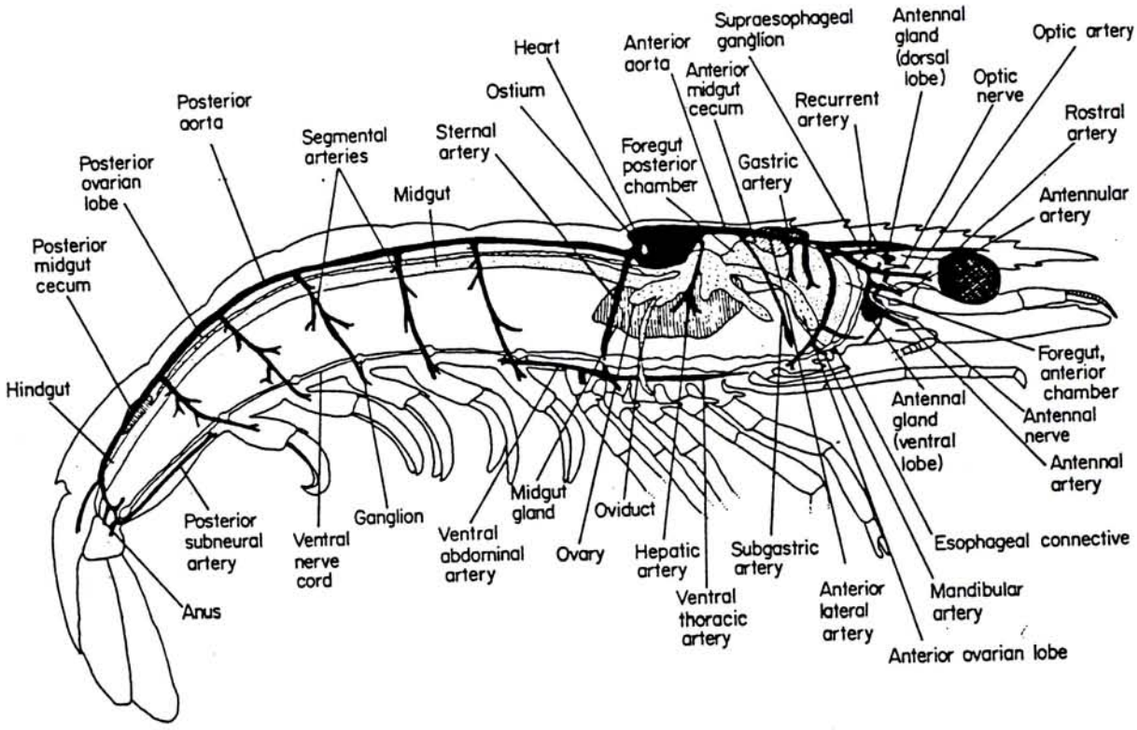



Fig. 2.1 Diagrammatic penaeid shrimp with gills and musculature removed to show major organ systems (from McLaughlin, 1980).

provides hemolymph to the eyes and supraesophageal ganglion. Anterior lateral arteries are the primary suppliers of hemolymph to the cephalic appendages, the walls of the foregut, the musculature, antennal glands, and carapace. Hepatic arteries supply hemolymph to the hepatopancreas. The posterior aorta provides the principal hemolymph supply to the abdomen, while the sternal artery supplies hemolymph to the walking legs, ventral nerve cord and anterior appendages including the scaphognathites. The arteries subdivide into arterioles and finally into capillary-like vessels bathing the tissue. The hemolymph contains the blue respiratory pigment hemocyanin, blood proteins, amino acids, lipids and carbohydrates. The circulatory system distributes nutrients, respiratory gases, metabolic wastes, and hormones throughout the body. Venous return occurs via distinct channels to the gills or branchiostegal membrane circulation, then to the pericardial cavity and finally to the heart.

### 2.1.2 Control mechanisms of cardiac function

The mechanisms controlling the cardiovascular system in crustaceans include intrinsic and extrinsic control (Wilkens and McMahon, 1992, review). Intrinsic control involves the neuronal output of the cardiac ganglion. A variety of extrinsic controls, both neural and neurohormonal, are also imposed on the heart.

#### 2.1.2.1 Intrinsic control

The heart of decapod crustaceans consists of a muscular ventricle suspended by a three-dimensional array of alary ligaments and arteries within the pericardial cavity. The ventricle is composed of striated muscle fibers which are multiple-innervated and capable of producing regenerative action potentials. The adult heart is neurogenic with primary excitation arising from the cardiac ganglion which is made up of a small number of autorhythmic (pacemaker) neurons (Alexandrowicz, 1932; Maynard, 1961; Horridge, 1965). The neuronal drive is supplied by 9 to 16 neurons. In isopods and decapods, the cardiac ganglion is located on the inner dorsal wall of the heart. In stomatopods, the cardiac ganglion is located on the outer dorsal wall of the heart. Muscle excitation is initiated by a group of large ganglionic cells acting as motoneurones. The length of systolic contraction depends upon the duration of the ganglionic burst (Anderson and Cooke, 1971; Hawlett, 1971; Benson, 1981). During systole, part of the energy is stored in the stretched elastic suspensory ligaments. During diastole this energy acts to restore heart volume and hemolymph enters the heart via the ostial valves.

#### 2.1.2.2 Extrinsic control

The heart receives extrinsic input from the central nervous system via the paired cardio-regulatory nerves as well as neurohormones released from the pericardial organs located on the lateral walls of the pericardial cavity (McMahon *et*

*al.*, 1997, review). In both isopods and decapods, the heart is innervated by two pairs of cardioacceleratory nerves and one pair of cardioinhibitory nerves which arise from the subesophageal ganglion in Macrura, or from homologous regions of the thoracic ganglion in Brachyura. In isopods, the cardioinhibitory nerves synapse on both the cardiac ganglion and on the myocardium while in decapods they only innervate the cardiac ganglion. Neurohormones represent a second route for extrinsic regulation of heart function. Hormones stored in and released from the pericardial organs include acetylcholine, dopamine, 5-hydroxytryptamine (5-HT), octopamine and the peptides proctolin, crustacean cardioactive peptide and several FMRFamide-related peptides.

Although crustaceans lack the arteriolar smooth muscle upon which much of the peripheral circulatory control of the vertebrate closed circulatory systems depends, cardioarterial valvular mechanisms under neural and neurohormonal control appear to be capable of selective distribution of cardiac output between the several separate arterial systems (McMahon and Burnett, 1990, review). Kuramoto and Ebara (1984) showed that cardioarterial valves are sensitive to octopamine and proctolin, but not to serotonin.

## 2.1.3 Ontogenic changes in cardiac function

### 2.1.3.1 Change in heart rate during development

Much is known about the function of the cardiovascular system in adult crustaceans but very little information is available on the early life history stages. In the brine shrimp *Artemia fransciscana*, the onset of cardiac function occurred in newly hatched individuals (Spicer, 1994). The relationship between heart rate and body weight of individuals of a wide range of sizes and ages did not form a linear relationship. Instead there was an initial rapid increase in rate early in cardiac ontogeny, followed by a slower decline with increasing body weight. Once the heart started beating, the heart rate increased with increasing body size and concomitant differentiation of cardiac tissue. However, when differentiation approached completion and cardiac growth had switched to elongation, there was a change in the pattern of cardiac function. There was then a decrease in heart rate with increasing body size, i.e., the heart rate showed an inverse relationship with body weight. In the water flea *Daphnia magna*, the amphipod *Gammarus duebeni*, the lobster *Nephrops norvegicus* (Spicer and Morritt, 1996) and the crayfish *Procambarus clarkii* (A. Wojciechowski and B.R. McMahon, in preparation), there was also a rapid increase in heart rate following the onset of cardiac functioning. This was followed by a less pronounced decrease in heart rate with continuing development.

### 2.1.3.2 Change from myogenic heart to neurogenic heart

The myocardium of adult crustaceans is not spontaneously electrically or mechanically active, but rather is driven by the nervous output of a cardiac ganglion (see 2.1.2.1). However, there have been studies suggesting that crustacean hearts are myogenic very early in development and later become neurogenic (Yamagishi, 1990; Yamagishi and Hirose, 1992). In the lobster *Homarus americanus*, the heart begins to pulse rhythmically in 4 to 5 week-old embryos, long before nervous innervation is established (Herrick, 1909). Yamagishi (1990) also suggested that the heart in early juveniles of the isopod *Ligia exotica* is myogenic, but this myogenicity becomes subordinate to neural drive during development. In the study on embryonic heart development in the crayfish *Procambarus clarkii*, a stimulus response was first observed during the 6$^{th}$ cardiac developmental stage as evidenced by a period of cardiac arrest lasting a few milliseconds (Wojciechowski and McMahon, in preparation). This reflex response to vibration suggests the presence of a coordinated neural system capable of perceiving changes in the environment. The results indicate that the heart of *P. clarkii* is myogenic before the 6$^{th}$ cardiac developmental stage and later becomes neurogenic. The earlier work by Carlson and Meek (1908) on the horseshoe crab *Limulus* showed that the heart was initially excited myogenically but pacemaker activity was gradually taken over by the developing cardiac ganglion. Thus this change from a myogenic heart to neurogenic heart is not limited to crustaceans but may apply to other arthropods. In order to explore the possibility of a link between cardiac innervation and changes in cardiac

function during embryonic development, there is a need of investigation on heart innervation in developing crustaceans.

## 2.2 Effect of Temperature on Crustacean Cardiac Function

### 2.2.1 General effect of temperature on crustaceans

Temperature limits the distribution of living organisms and is an important determinant of their activities. Maintenance of effective organismic integrity by regulating the balance between the rates of various biochemical activities is a very important aspect for survival. The body temperature of most poikilotherms is dependent upon the environmental temperature. Certain environments provide nearly constant thermal conditions and permit poikilotherms to maintain stable body temperatures. Physiological and behavioral observations made on poikilotherms from such thermally stable environments have revealed limited tolerance to sudden temperature change (Florey and Hoyle, 1961, 1976; Hammel *et al.*, 1967). In contrast, other poikilotherms which live in environments with dramatic thermal fluctuations, both short term and long term, must tolerate and adapt to temperature changes in order to maintain functional integrity (Stephens and Atwood, 1982). Temperature affects the total metabolism of the body which in turn affects circulation, ventilation, oxygen consumption (Cameron and Mangum, 1983; Wilkens and McMahon, 1992, reviews) and hemolymph pH (Truchot, 1978).

### 2.2.2 Effect of temperature on heart rate

In general, heart rate increases with temperature as shown in a variety of crustaceans, including the shore crab *Carcinus maenas* (Ahsanullah and Newell, 1971; Aagaard, 1996), Dungeness crab *Cancer magister* (McMahon *et al.*, 1978; De Wachter and McMahon, 1996), blue crab *Callinectes sapidus* (Burton *et al.*, 1980), crab *Hemigrapsus sanguineus* (Depledge, 1984), crayfish *Cherax tenuimanus* (Villareal, 1990) and squat lobsters *Munida rugosa* and *M. sarsi* (Zainal *et al.*, 1992). However, in a number of cases, heart rate increases with temperature to a certain level and then levels off. The heart rate in *Cancer magister* and *C. productus* was found to increase with the temperature over the range of 0 to 20-22°C (Florey and Kiebel, 1974). Beyond this range no further increase was observed and at around 25°C the heart rate decreased. A study on the effect of acute temperature change on *Carcinus maenas* showed that heart rate increases with temperature up to approximately 25°C (Ansanullah and Newell, 1971). Heart rate levels off from 25 to 30°C. In the Australian crayfish *Cherax tenuimanus*, heart rate of animals maintained at 22°C was shown to be the lowest at 18°C and highest at the acclimation temperature (Villarreal, 1990). Heart rate became irregular at 26°C. Analysis of covariance showed that temperature was the principal factor among light intensity, salinity and depth in influencing the heart rate. In *Daphnia*, the relative enhancement of heart rate under turbulence increased with temperature up to a maximum and decreased with further temperature increment (Alcaraz *et al.*, 1994). Studies on semi-isolated heart and intact heart of the Dungeness crab, *Cancer magister* showed that as temperature increased from 4 to 20°C, heart rate in intact

animals showed a linear relationship with temperature up to 20°C (De Wachter and Wilkens, 1996). Heart rate of semi-isolated heart also showed a positive but different linear relationship with temperature up to about 18°C. At higher temperatures, this correlation disappeared in both semi-isolated and intact heart. Semi-isolated heart is a good model to observe the direct effects of perturbations such as temperature on an autorhythmic system. Semi-isolated heart refers to a heart that was freshly dissected out but left attached to the dorsal side of the carapace while the alary ligaments and pericardial septum were intact. The cardioregulatory nerves were cut and the preparation was washed with saline.

Acute exposure of squat lobsters *Munida rugosa* and *M. sarsi* originally kept at 10°C to temperatures between 5 and 15°C showed that, as in most other decapods, heart rate increased with temperature (Zainal *et al.*, 1992). There was a pronounced increase progressively up to 20°C. When both species were exposed to 20°C for up to 2 h, heart rate became irregular. At this stage, if the temperature was slowly reduced to normal (10°C), the animals usually recovered and heart rate returned to normal levels within a few hours (6-10 h). *M. rugosa* could survive after short periods (90 min) exposure to temperature as high as 20°C, but with increasing duration of exposure, there was an increase in mortality rate. Both species showed progressive decrease in heart rate at 25°C. It was clear that the animals were under stress at the higher temperatures which are outside the range normally experienced in their natural environment.

The temperature coefficient $Q_{10}$ is the ratio of reaction velocities at temperatures ten degrees Celsius apart. Many studies have used $Q_{10}$ to reflect the effect of temperature on reaction rates. In a variety of crustaceans, heart rate increases with temperature to yield $Q_{10}$ values between 4 at low temperatures and 1.5 at high temperatures (Maynard, 1960a, review; Ahsanullah and Newell, 1971; Florey and Kriebel, 1974; Spaargaren, 1974; Spaargaren and Achituv, 1977; McMahon, *et al.*, 1978; deFur and Mangum, 1979; Burton *et al.*, 1980; Depledge, 1984; McMahon and Burnett, 1990; Zainal *et al.*, 1992). The values indicate that reaction rates have a greater response to temperature change at low temperature. Besides, the reduction in $Q_{10}$ towards the upper temperature limit has also been interpreted as an indication that the rate of increase of metabolic rate with increase in temperature cannot be sustained (Varo *et al.*, 1991). In *Cancer magister* and *C. productus*, $Q_{10}$ for the range of 4 to 19°C is 2, below 4°C the apparent $Q_{10}$ is greater, and above 19°C it is smaller (Florey and Kiebel, 1974). The $Q_{10}$ values for heart rate obtained in *Munida rugosa* and *M. sarsi* confirm that the animals were under stress at temperatures close to 20°C (Zainal *et al.*, 1992). When animals were kept at this temperature for periods up to 21 h, heart rate became irregular. Prolonged exposure resulted in death. In contrast, both species survived for many hours at temperatures of less than 5°C. For both species, $Q_{10}$ between 10-15°C had values of approximately 2 but at a higher temperatures, there was a reduction in $Q_{10}$.

$Q_{10}$ can also indicate the temperature sensitivity at specific temperature range. Burton *et al.* (1980) showed that heart rate increases with temperature in the blue crab *Callinectes sapidus*. The degree of sensitivity to temperature decreases with

increasing temperature. In a study on temperature effect on the Dungeness crab *Cancer magister*, animals were kept at 12°C and exposed to stepwise temperature decrease from 12 to 4°C, or increase from 12 to 20°C (De Wachter and McMahon, 1996). The temperature range tested was selected to span almost the complete natural range of the crab. Results showed that there was a gradual decrease in $Q_{10}$ with values smaller than 1.8 in upper half of the temperature range. This represents a reduction in temperature sensitivity with increasing temperature. Another study on semi-isolated heart and intact heart of Dungeness crab, *Cancer magister* showed that acute $Q_{10}$ of the semi-isolated hearts was always below 2, and it decreased with increasing temperature (De Wachter and Wilkens, 1996). The $Q_{10}$ at the high temperature range was around 1. Heart rates of semi-isolated hearts recovered fully at 12°C regardless of whether temperature was increased or decreased first. In intact animals, the $Q_{10}$ decreased from 3 at 4°C to 1.5 at 20°C. The different $Q_{10}$ values in semi-isolated heart and intact heart suggested that semi-isolated heart has a lower temperature sensitivity.

### 2.2.3 Inter-relationship between heart rate, stroke volume and cardiac output

Adjustment of cardiac output can occur by variation of heart rate and/or stroke volume. Increase in heart rate may increase cardiac output but may also lead to a decrease in cardiac output, as a substantial decrease in stroke volume can lead to a decreased cardiac output despite an increase in heart rate. The decreased stroke

volume may be associated with incomplete filling of the heart at high heart rates. In *Cancer magister* and *C. productus*, heart rate of the crab increased with temperature but cardiac output declined (Florey and Kiebel, 1974). Study on semi-isolated heart and intact heart of *Cancer magister* showed that heart rate increases with temperature, but the cardiac output of intact heart increased while that of semi-isolated heart decreased (De Wachter and Wilkens, 1996).

### 2.2.4 Control mechanism on crustacean cardiac function in response to temperature change

The moderation of heart rate in response to temperature changes may involve both intrinsic and extrinsic control mechanisms. The intrinsic controls include the direct effect of temperature on heart rate and also the increased contractions resulting from double neural bursts in cardiac ganglion (De Wachter and Wilkens, 1996). The extrinsic control systems include central nervous mechanisms operating via the cardioregulatory nerves and changes in circulating levels of neuroactive substances. Studies on the responses of semi-isolated hearts reveal the effect of temperature on the intrinsic pumping performance of the heart, that is, the effect of temperature on cardiac ganglion (De Wachter and Wilkens, 1996). On the other hand, studies on intact animals reveal the effect of temperature on cardiac ganglion as well as cardioregulatory nerves and neurohormones. This assumption is supported by the observations of different cardiac responses to neurohormones in semi-isolated hearts and in intact crabs (McGaw *et al.*, 1995). Semi-isolated heart is a good model to

observe the direct effects of perturbations such as temperature on an autorhythmic system. Heart rates in semi-isolated hearts are lower than in intact animals. Besides the lower heart rate, the lower $Q_{10}$ values for semi-isolated hearts show that these preparations are less responsive to temperature change than the heart of intact animals. The values of heart rates and $Q_{10}$ suggested that control in semi-isolated heart is via the cardiac ganglion. Yet in intact animals, the cardiac ganglion is only partially responsible for the temperature dependence of the heart rate. Other control mechanisms such as change in cardioregulatory nerves stimulation and level of circulating neurohormones may change in response to temperature and thus may play a role in control of cardiac function.

In *Cancer magister* and *C. productus*, temperature was shown to affect the burst pattern of the cardiac ganglion (Florey and Kiebel, 1974). There is a change in the mode of activation of the heart muscle associated with a change in temperature. Both the force of contraction and the degree to which the myocardium has been stretched by diastolic filling would affect the resulting stroke volume. At low heart rate, filling may be more complete, so that smaller and less frequent action potentials, as seen at lower temperatures, may still cause a rather large stroke volume while incomplete filling at high heart rates may lead to relatively smaller stroke volumes (Florey and Kiebel, 1974). The decrease in stroke volume with increasing temperature related to the decrease in ventricular pressure and systolic duration was seen in semi-isolated heart of *Cancer magister* (De Watcher and Wilkens, 1996). The decrease in ventricular pressure may be due to a combination of the reduction in cardiac ganglion input to the myocardium and the direct negative effects of elevated

temperature on muscle contractility. The results suggested that the spike firing frequency must have decreased with increasing temperature. This is supported by a study by Jacobs and Atwood (1981) that a decrease in neural bust frequency is correlated with decreased tension in the claw opener muscle of the crayfish. Similarly, increased temperature induced a reduction of muscular tension in the chelae muscles of different crustaceans (Fisher and Florey, 1981; Jacobs and Atwood, 1981; Stephens and Atwood, 1982). Additionally, a conformational change in the isomerization of the actomyosin complex, which is hypothesized to generate movement, is highly temperature dependent (Taylor, 1991). Moreover, increase in temperature leads to a change in membrane fluidity. This may influence the ion channels in the membranes and alter intracellular potassium and calcium levels (Prosser, 1991). Above 20°C, heart rate of the semi-isolated hearts of *Cancer magister* becomes irregular, ventricular pressures and cardiac ganglion input to myocardium drop sharply and approach zero, and stroke volume and cardiac output fall to zero. These measurements suggest that, although the rhythm generator still works at these temperatures, the motoneuronal bursts are more and more poorly translated into contractions of the cardiac muscles. The disappearance of contraction in at least short exposure to high temperatures is not related to structural damage to enzyme systems, because a decrease in temperature restores the heart to its original functionality.

The extrinsic controls that can modulate the burst rate of the cardiac ganglion may include the input from cardioregulatory nerves and the neurohormones such as octopamine and serotonin (McGaw *et al.*, 1994, 1995). Stimulation of the

accelerator nerves in crayfish results in an increase in heart rate (Florey, 1960; Wilkens and Walker, 1992). Therefore, the difference in heart rate and $Q_{10}$ between intact *Cancer magister* and the semi-isolated hearts in response to temperature probably arises from modulation of the cardiac ganglion by cardioregulatory nerves as well as neurohormones (De Wachter and Wilkens, 1996). The specific role of each of the possible extrinsic control systems is still unclear. The roles played by the different neuroactive substances and their temperature dependence, and the temperature effects on the cardioregulatory nerves remain to be elucidated.

## 2.3 Effect of Salinity on Crustacean Cardiac Function

### 2.3.1 General effect of salinity on crustaceans

Salinity is an important environmental factor governing the distribution of animals in coastal waters, particularly in estuaries, salt marshes and coastal lagoon. In an estuary, periodic changes in salinity are caused by the tide. When the water rises in the estuary, a salt-wedge is moving in the direction of the land. Its passage will cause a sudden increase in salinity in the water layer just above the bottom. With a falling tide, a decrease in salinity over the bottom will occur. Moreover, variation in salinity also results from desiccation and rainfall. Its has long been known that salinity affects the hatching, development, survival and distribution of crustaceans (Mantel and Farmer, 1983, review). Changes in salinity are associated

with changes in osmolarity and ionic concentration of hemolymph and in the oxygen affinity of hemocyanin.

The process of osmoregulation involves metabolic work and is associated with an increase in oxygen demand. Some crustaceans are osmoconformers that can tolerate change in salinity to a large extent and only osmoregulate in stressful range. When *Carcinus maenas* was transferred from 100% SW (seawater) to dilute medium, osmoregulation was only observed after the environmental medium fell below 75% (Shaw, 1961a). Thus *C. maenas* is an osmoconformer between 100% and 75% seawater SW; below 75% SW, the crab begins to hyperregulate. Taylor (1977) also showed that *C. maenas* partially osmoregulates in hyposmotic media in such a way that it keeps its hemolymph hyperosmotic to the environment whilst tolerating some internal dilution. Similar results were reported in the euryhaline amphipod *Gammarus duebeni* (Kinne, 1952) and euryhaline shrimp *Crangon crangon* (Spaargaren, 1973). Meanwhile, the lobster *Homarus americanus* is a limited osmoregulator below 20‰ (Jury *et al.*, 1994a). *H. americanus* allows its hemolymph osmolarity to drop as the environmental salinity is reduced, but always maintaining it higher than the ambient osmolarity.

Crustaceans utilize a number of different mechanisms to maintain the internal osmotic and ionic environment. Osmotic and ionic regulation in crustaceans has been widely studied (Prosser, 1973; Mantel and Farmer, 1983; reviews). Active mechanisms include avoidance behavior and active pumping of ions via $Na^+$, $K^+$-ATPase and other enzymes across the gills and other surfaces (Krogh, 1938, Green *et*

*al.*, 1959, Shaw, 1961a). Passive mechanisms include decreased cuticle permeability so as to limit the diffusion of water and ions across body surfaces (Smith, 1967, 1970; Shaw 1961b; Rudy, 1967; Potts and Parry, 1964), production of organic osmolytes (e.g., amino acids) and their release into the hemolymph and the control of the volume and /or ionic concentration of the urine (Green *et al.*, 1959; Shaw, 1961a). Some of the above processes appear to be controlled by hormonal mechanisms, especially in crustaceans living in habitats with salinity variation (Hume and Berlind, 1976).

The relationship between osmoregulation in hyper-tonic or hypo-tonic media and energy consumption has intrigued many research workers. Maintaining a difference in osmotic concentration between body fluid and medium requires energy, which has to be supplied by active processes, linked with metabolism. For a great number of aquatic animals, the relation between oxygen consumption and the difference in osmotic concentration maintained by the animal between the body fluid and environment was investigated. From the differences in oxygen consumption in animals under different osmotic stresses, the energy consumption of osmoregulating processes can be estimated (Potts, 1954, review). In a number of cases, a clearly positive correlation was found between osmotic performance and oxygen consumption. In other cases no relation or even inverse ratio seemed to exist.

### 2.3.2 Effect of salinity variation on heart rate

The effect of changes in environmental salinity on the rate of oxygen consumption has been studied in several species of decapod crustaceans (Kinne, 1971). Although there are some variations between species, most species show an increase in the rate of oxygen consumption in response to increase or decrease in salinity. Table 2.1 shows the effects of decreasing salinity on the metabolic rate of crustaceans (Jury *et al.*, 1994b). Increase in energy consumption is necessary for actively pumping ions and avoidance behavior. The increase in oxygen consumption imposed by salinity stress can be adjusted by the cardiovascular system via change in cardiac output, heart rate, stroke volume and hemolymph flow distribution, and by the respiratory system via change in scaphognathite rate and ventilation volume. To deal with the increase in oxygen consumption, different animals employ different strategies. Some animals show an increase in heart rate while some show a decrease in heart rate. However, other animals may show no response. For example, Taylor *et al.* (1977) reported no change in heart rate when *Carcinus maenas* was exposed to 50% seawater. *Crangon crangon* responds to medium dilution with an increased ventilation but not with an altered heart rate (Dyer and Uglow, 1980). This apparent unresponsiveness of the heart contrasts with other reports on decapod responses to reduced salinity.

Exposure of shore crab *Carcinus maenas* to a reduction in salinity is accompanied by an increase in oxygen consumption and heart rate (Hume and Berlind, 1976; Taylor, 1977). Since the oxygen transporting properties of the

Table 2.1 Effects of decreasing salinity on the metabolic rate of crustaceans (Jury *et al.* 1994b).

| Oxygen consumption | | | Species | Source | Remarks |
|---|---|---|---|---|---|
| Change in salinity (‰) | Change in ml $O_2$/g/h | % increase per 10 ‰ | | | |
| 30 to 5 | 0.0093 to 0.0178 | 36.5 | *Panoperus herbstii* | Dimck & Groves, 1975 | acclimated to 30 ‰, 10°C |
| 30 to 15 | 0.205 to 0.293 | 28.6 | *Penaeus japonicus* | Chen & Lai, 1993 | Juveniles at 15°C |
| 37 to 10 | 0.2 to 0.4 | 37.0 | *Penaeus japonicus* | Dalla Via, 1986 | Approx. values |
| 34 to 12 | 0.03 to 0.052 | 30.3 | *Carcinus maenas* | Taylor, 1977 | Approx. values |
| 30 to 15 | 0.028 to 0.036 | 19.0 | *Carcinus maenas* | Taylor *et al.*, 1977 | Tested at 14°C |
| 26 to 2 | 0.072 to 0.148 | 44.0 | *Callinectes rathbounae* | Rosas *et al.*, 1989 | |
| 35 to 5 | 1.6 to 3.4 | 37.5 | *Callinectes sapidus* | Engel & Eggert, 1974 | Approx. values from excised gill |
| 30 to10 | 0.138 to 0.175 | 13.4 | *Callinectes sapidus* | Findley *et al.*, 1978 | Approx. values acclimated to 30 ‰, 20°C |

hemolymph of *Carcinus maenas* show little change under conditions of reduced salinity, the increased oxygen demand of the tissues is met by a rise in cardiac output resulting mainly from an increase in heart rate. Reduction of salinity from 34 to 20‰ resulted in an almost immediate increase in the rate of oxygen consumption. Changes in heart rate also correlated with changes in oxygen consumption. DeFur and Mangum (1979) also showed that *Palaemon serratus* (hypo-regulating) and *Crangon crangon* (hyper-regulating) had a slight increase in heart rate at both hypo- (60% SW) and hyper- (120% SW) osmotic environment. The increase in heart rate was accompanied by increase in oxygen uptake. Similar results were reported in isopods *Idotea balthica*, *I. emarginata*, *I. neglecta,* mysid *Praunus flexuosus,* estuarine copepods *Acartia tonsa*, *Eurytemora hirundoides* and shrimps *Crangon vulgaris* and *Metapenaeus monoceros* (Kinnes, 1967, review). Heart rate of the lobster *Homarus americanus* increased when transferred from 20 to 15‰ and then to 10‰ (Jury *et al.*, 1994b). McGaw and McMahon (1996) showed that short term exposure (6 h) of the Dungeness crab *Cancer magister* to 50% SW resulted in increased heart rate. Initial levels were regained only slowly on return to 100% SW.

The increase in heart rate at low salinity presumably increases cardiac output and thus enhances oxygen transport to vascularized tissue and a parallel increase in ventilation provides additional oxygen to vascularized tissue. Thus, the observed increase in oxygen uptake in response to salinity stress involves both the cardiovascular and ventilatory systems (DeFur and Mangum, 1979). Heart rate may entail a certain blood pressure. For water transport, this hydrostatic pressure is equivalent to osmotic pressure. With hypertonic regulation, an increased heart rate

may restrict the osmotic inflow of water by increasing the blood pressure (DeFur and Mangum, 1979). For strong hypo-regulating *Palaemon serratus* and faintly hyporegulating species *Crangon crangon*, if increase in blood pressure does not occur, it would mean an extra loss of water by ultrafiltration. It could be deduced that increase in heart rate at hypernormal salinities would probably be connected only with active salt excretion. The slight difference maintained between osmotic concentration of the hemolymph and that of the environment increases both at salinities higher or lower than the ionic concentration of the hemolymph, suggesting that an increase in heart rate always corresponds with an increase in osmotic difference between hemolymph and environment.

Most studies on salinity effect on crustaceans showed that an increase in heart rate was employed by animals which encounter salinity stress. However, a decrease in heart rate was noted in some studies. The stenohaline crab *Libinia emarginata* showed a decrease in heart rate when transferred from 100% to 80% SW (Cornell, 1973, 1974). The slight difference maintained between osmotic concentration of the hemolymph and that of the environment decreased at hypernormal salinities. Long term exposure (4 days) of *Cancer magister* to 50% SW showed that heart rate decreased (McGaw and McMahon, 1996). Also, the shrimp *Lysmata seticardata*, an osmoconformer, had a lower heart rate at higher salinity (100 & 120%SW) (Spaargaren, 1973). These studies demonstrated that a decrease in heart rate may also serve to compensate for salinity changes. In general, gill ventilation rate increases and heart rate decreases as $PO_2$ declines below normal environmental levels (DeFur and Mangum, 1979). Oxygen consumption is

maintained at $PO_2$ values above the species-dependent critical oxygen tension (Pc), which is affected by other environmental factors such as temperature and salinity as well as endogenous factors including the ability to supply oxygen to the tissues. Below the Pc, ventilation and oxygen consumption rates decline and heart rate is further reduced since there is insufficient oxygen available to meet the aerobic demands of the respiratory and circulatory pumps. Decreases in heart rate might partially account for the decrease in water permeability of the body surface. Decline in heart rate results in a corresponding decrease in the flow of hemolymph through the gill. This could reduce the average exchange gradient for inward movement of water and outward ion flux resulting from low salinity stress. Moreover, decrease in heart rate permits adjustments of hemolymph $PO_2$ that enhance the respiratory role of hemocyanin in the altered ionic environment in the hemolymph.

### 2.3.3 Effect of salinity on hemolymph flow distribution

In addition to a change in heart rate, a change in hemolymph flow distribution may also be able to meet increased local oxygen demand imposed by salinity stress. This could be done by a combination of high hemolymph residence time and increased ventilatory water flow, allowing high oxygen saturation of brachial excurrent hemolymph and increased oxygen extraction from the circulating hemolymph. Study on the effect of low salinity on *Cancer magister* showed that hemolymph flow through sternal artery and posterior aorta increases (McGaw and McMahon, 1996). The increase of flow in sternal artery possibly reflecting the

increased metabolic demand involved in halokinesis and regulation of the internal ionic concentration. The redistribution of hemolymph during hypo-osmotic exposure could be controlled by the direct ionic action on cardioregulatory nerves (DeFur and Mangum, 1979) or more likely, by the release of cardiac neurohormones from the pericardial organs. Several pericardial hormones have been shown to modulate heart rate and alter hemolymph flow in *Cancer magister* (Airriess and McMahon, 1992; McGaw *et al.*, 1994) possibly by their direct action on the heart or cardioarterial valves as indicated by the work on the lobster *Homarus americanus* (Kuramoto and Ebara, 1984, 1989). Since the posterior aorta supplies hemolymph to local tissues of the hindgut, the increase of the posterior aorta flow in *Cancer magister* when subjected to low salinity suggested that hindgut may also be involved in ionic regulation (McGaw and McMahon, 1996). Phillips *et al.* (1986) showed that rectum and ileum of insects are involved in ionic regulation. *Schistocerca gregaria* ion-transport peptide (Scg-ITP), the neuropeptide which influences selective ionic transport in the locust ileum is found to be structurally related to a crustacean neuropeptide family including the crustacean hyperglycaemic hormones from *Carcinus maenas* and the crayfish *Orconectes limosus*, and molt-inhibiting hormone and vitellogenesis inhibiting hormone from the lobster *Homarus americanus* (Audsley *et al.*, 1992). It is possible that neurohormones play a similar role in ion transport in crustaceans. Although the patterns of heart and hemolymph flow rates do not correspond to any one hormone tested so far, it is possible that they could result from the release of a combination of neurohormones (McGaw and McMahon, 1996).

# CHAPTER 3

# CHANGES IN HEART RATE DURING DEVELOPMENT

## 3.1 Introduction

Functional development of the cardiovascular system in invertebrates is poorly understood (McMahon *et al.*, in press). Information on the development of heart function is only available in a few crustaceans. In the brine shrimp *Artemia franciscana* and the water flea *Daphnia magna*, the onset of cardiac function is post-hatch (Spicer, 1994; Spicer and Morritt, 1996). However, in the amphipod *Gammarus duebeni*, the lobster *Nephrops norvegicus* (Spicer and Morritt, 1996) and the crayfish *Procambarus clarkii* (Wojciechowski and McMahon, in preparation), cardiac function commences prior to hatching. Earlier studies on the relationship between heart rate and body weight in crustaceans of widely differing body weight showed that heart rate decreases with body weight (Maynard, 1960b). However, Spicer and Morritt (1996) and Wojciechowski and McMahon (in preparation) demonstrated that the relationship between heart rate and body weight is more complex in crustacean larval development. There is a period in which heart rate increases despite the increase in body weight, followed by a period in which heart rate decreases with increased body weight (McMahon *et al.*, 1997). Among the few studies on the development of cardiac function, the lobster *Nephrops norvegicus* (Spicer and Morritt, 1996) and the crayfish *Procambarus clarkii* are the only

decapod crustaceans studied. Both of them are members of the suborder Pleocyemata in which the females carry the eggs under the abdomen and the eggs hatch as zoea or later stages (Williamson, 1982). No animals in the other suborder, Dendrobranchiata, in which the female directly releases the eggs in the water and the eggs hatch as nauplii, have been studied. Since the larval development in Dendrobranchiata is more complicated than that in Pleocyemata, a different, more complicated pattern of change in heart rate may occur. In the present study, *Metapenaeus ensis,* a penaeid shrimp of the suborder Dendrobranchiata, was chosen as the experimental animal. Besides the change in heart rate during larval development and its relationship with body weight, the change in control of heart pulsation is also an interesting topic. There have been studies suggesting that crustacean hearts are myogenic early in development and later become neurogenic. Examples are the lobster *Homarus americanus* (Herrick, 1909), the isopod *Ligia exotica* (Yamagishi, 1990) and the crayfish *Procambarus clarkii* (Wojciechowski and McMahon, in preparation).

The first objective of this study is to examine the change in heart rate during larval development of *Metapenaeus ensis*, and to investigate its relationship with body weight. Part of the results on the change in heart rate during development of *Metapenaeus ensis* has been reported (McMahon *et al.*, 1995). The second objective of the study is to investigate the possible stage at which the heart changes from myogenic to neurogenic.

## 3.2 Materials and methods

Gravid *Metapenaeus ensis* females were acquired from local market and maintained in individual 500-l fiberglass tanks containing well-aerated filtered seawater from Tolo Harbor. Ethylene-diaminetetraacetic acid, (EDTA), disodium salt, was added at a concentration of 10 mg/l as a chelating agent to reduce toxic effect of metal ions. The shrimp usually spawned in the early hours of the following day and the eggs hatched after about 12 hours. *Metapenaeus ensis* has six naupliar stages (NI to VI), three protozoeal stages (PZI to III) and three mysid stages (MI to III) (Leong *et al.*, 1992). Larval development is completed in 9 to 10 days. After metamorphoses, they become postlarvae (PL). At PL day 1 to 5, they are planktonic. From PL day 5-6 onwards, they become benthic.

After the shrimp reached the protozoea I (PZI) instar, they were transferred to 10-l aquarium at a density of about 100 individuals per liter. The temperature was maintained at 28°C by an aquarium heater. Oxygenation and agitation were provided by gentle air bubbling. In larval stages, the shrimp were fed with the diatom *Chaetoceros gracilis* at a density of about $10^6$ cells $ml^{-1}$. When they became postlarvae, they were fed with rotifers at density of 4000 individuals $l^{-1}$ or *Artemia* nauplii at a density of 450 individuals $l^{-1}$.

To study the onset of cardiac function, shrimp were observed at NI and each subsequent stage. To study the change in heart rate during larval development, heart rate of shrimp at different developmental stages was measured using micro-

videophotography. Since heartbeat was initiated at NVI, heart rate of shrimp at nauplius VI (NVI), protozoea I (PZI), protozoea II (PZII), protozoea III (PZIII), mysis I (MI), mysis II (MII), mysis III (MIII), planktonic postlarvae (PL day 2 to 4 [PL(p)]) and benthic postlarvae (PL day 7 to 9 [PL(b)]) was measured. All measurements were done at room temperature (26±2°C). The shrimp were put on a depression slide and held in position by the cotton wool, and then examined under the microscope (Nikon SMZ-2T). The microscope was connected to the video camera (Panasonic WV-1550). The video signal was synchronized with a time-date generator (Panasonic WJ-CD 810) and recorded on a video cassette recorder (Panasonic AG 7300). The heart rate was recorded within 2 minutes after placing the shrimp on the depression slide to minimize the hypoxic and temperature effects on heart function. This had to be done because the heart rate was found to increase in response to hypoxic effect and change with temperature. Preliminary studies have shown that the heart rate measured in this way is not different from the rate measured in animals in a chamber continuously perfused with seawater as described in 4.2. The heart rate was very high so that determination was done during slow playback. The number of heartbeat in five seconds was measured. To investigate the possible stages when the heart of *Metapenaeus ensis* becomes neurogenic, a great hit was applied on the bench during measurement of heart rate at each developmental stage.

To study the change in body weight during larval development, 4 batches of shrimp, each with 10 animals of stages NVI, PZI, PZII, PZIII, MI, MII, MIII, PL(p) and PL(b) were washed gently with distilled water to remove the salt on body surface. After that, the shrimp were put into pre-weighed aluminum vials and dried

at 60°C for 10 hours and the weight was measured to the nearest 0.1 μg with a micro-balance (CAHN C-31).

## 3.3 Results

The heartbeat of *Metapenaeus ensis* was first observed at NVI stage. From stage NI to NV, no heart-like structure or beating structure was observed. Heart rate increased slowly as the shrimp grew. Stages NVI, PZI and PZII showed non-rhythmic heart rate. The heart did not beat regularly until the shrimp reached PZIII instar. The results on heart rate are shown in Fig. 3.1. Heart rate of the shrimp increased from 84.3 ± 9.7 beats $min^{-1}$ at NVI to a peak of 554.4 ± 10.9 beats $min^{-1}$ at PZIII. The heart rate then decreased from 504.9 ± 16.5 beats $min^{-1}$ at MI to 357.3 ± 10.2 beats $min^{-1}$ at PL(b). Besides the change in heart rate, a change in heart shape was also observed during larval development (Fig. 3.2). Observation from dorsal side of the heart showed that the heart first appeared as kite-shape at NVI and protozoeal stages. At mysid stages, the posterior end of the heart became flattened. At postlarvae, the heart became rectangular in shape, similar to that of the adult.

Starting from stage MII and MIII, heartbeat of the shrimp would stop for a few milliseconds in response to a great hit applied on the bench during measurement of heart rate. This response suggested that their heart was neurogenic that it could sense the vibration and give responses. However, in the earlier stages, no such responses occurred which implies the heart was myogenic that could not sense the

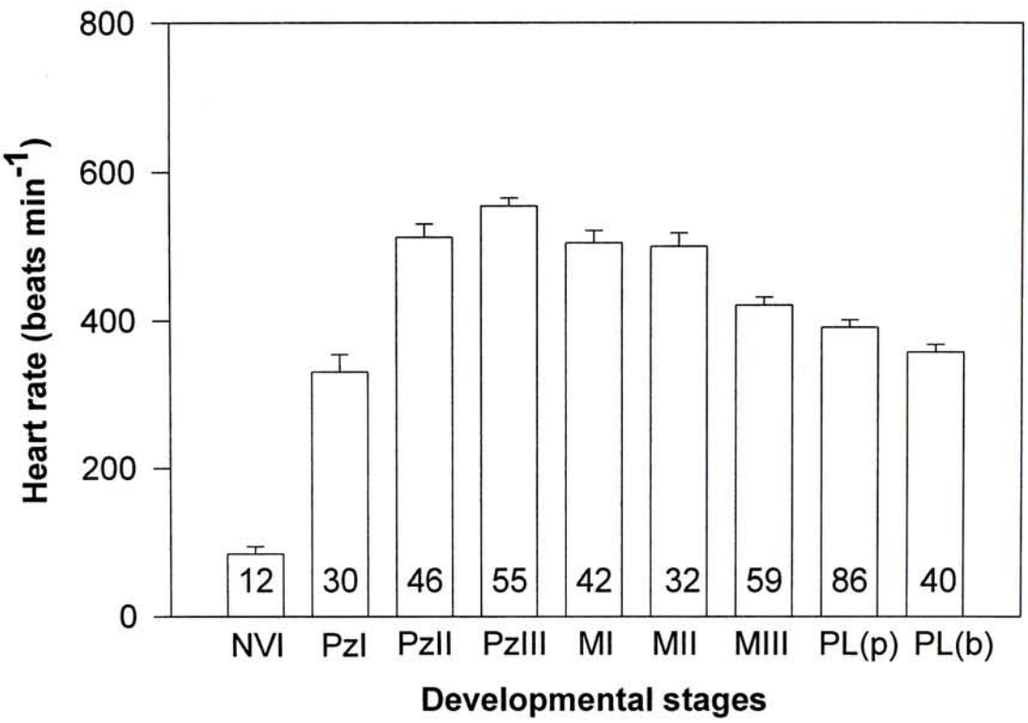
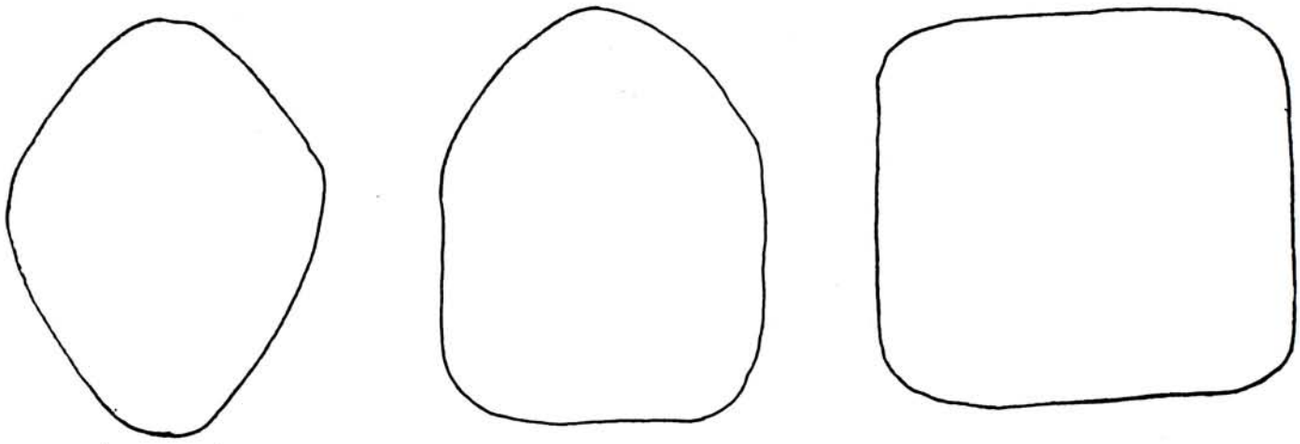



Fig. 3.1 Change in heart rate during development of *Metapenaeus ensis*. NVI: nauplius VI; PZI to III: protozoea I to III; MI to MIII: mysis I to III; PL(p): planktonic postlarvae (postlarvae day 2 to 4); benthic postlarvae (postlarvae day 7 to 9). Values are means ± standard error. The numbers inside the bar represent the sample size.



[a] [b] [c]

Fig. 3.2 Change in heart shape during development of *Metapenaeus ensis* (the drawings are the dorsal view of the heart and they are not to scale). [a] Heart shape of NVI and protozoeal stages. [b] Heart shape of MI, MII and MIII. [c] Heart shape of postlarvae.

stimulus and give response. Therefore, the suspected transitional stage from myogenic heart in earlier stages to neurogenic heart in adult is MII to MIII.

The results on body weight are shown in Fig. 3.3. Body weight of shrimp increased from 9.0 ± 0.7 μg at NVI to 86.8 ± 5.3 μg at PL(b). The relationship between heart rate and body weight during larval development is a biphasic form (Fig. 3.4). The heart rate (F) increased with body weight (X) from NVI to PZIII ($F = 0.56\ X^{1.70}$, $n = 4$, $r = 0.91$, $0.10 > P > 0.05$) but then decreased with body weight from PZIII to benthic postlarvae ($F = 3.20\ X^{-0.34}$, $n = 6$, $r = 0.97$, $P < 0.005$).

## 3.3 Discussion

In *Metapenaeus ensis* heartbeat is first observed in the last naupliar instar. The heart showed non-rhythmic beating until the shrimp reached PZIII instar. Similarly, slow and irregular heart rate commencing at about the third naupliar stage was reported in *Artemia franciscana* (Spicer, 1994). However, in two decapods studied, viz. the lobster *Nephrops norvegicus* (Spicer and Morritt, 1996) and the crayfish *Procambarus clarkii* (Wojciechowski and McMahon, in preparation), cardiac function commenced prior to hatching. The difference may be related to the difference in larval development between penaeids (suborder Dendrobranchiata) and the lobster/crayfish (suborder Pleocymata). In the later, the eggs hatch as zoea or later stages, which are more advanced than the nauplius larvae (Williamson, 1982). The mysid stage in the penaeids is believed to be equivalent to zoea stage in the

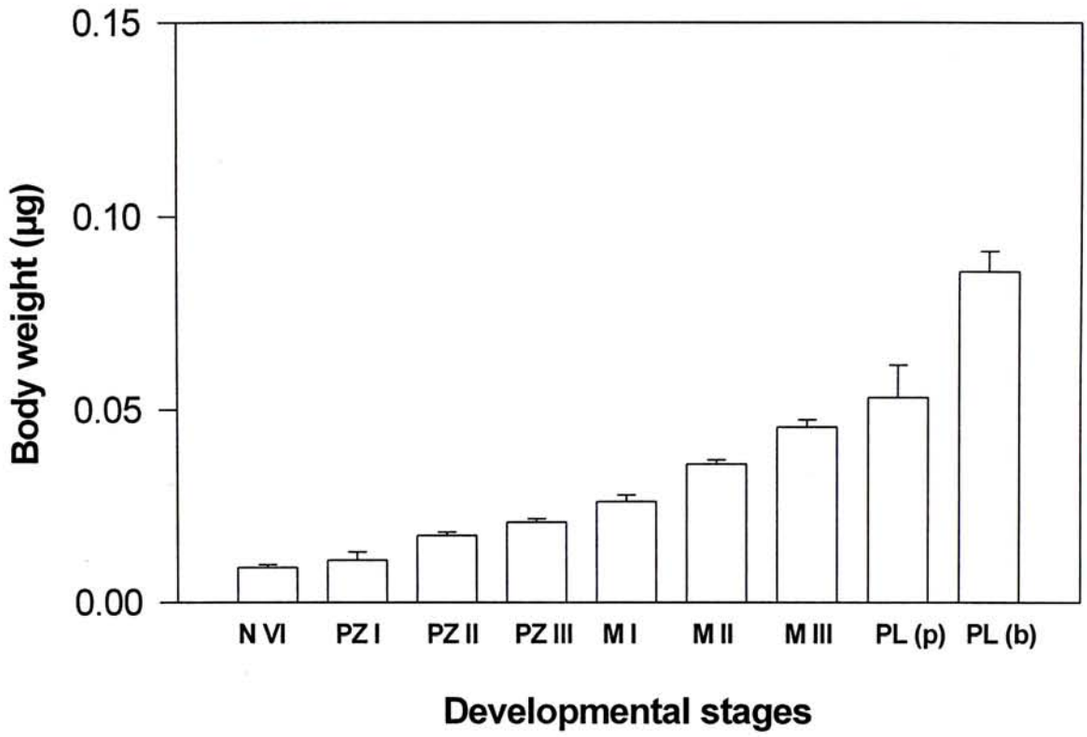



Fig. 3.3 Change in body weight during development of *Metapenaeus ensis*. NVI: nauplius VI; PZI to III: protozoea I to III; MI to MIII: mysis I to III; PL(p): planktonic postlarvae (postlarvae day 2 to 4); benthic postlarvae (postlarvae day 7 to 9). Values are means ± standard error.

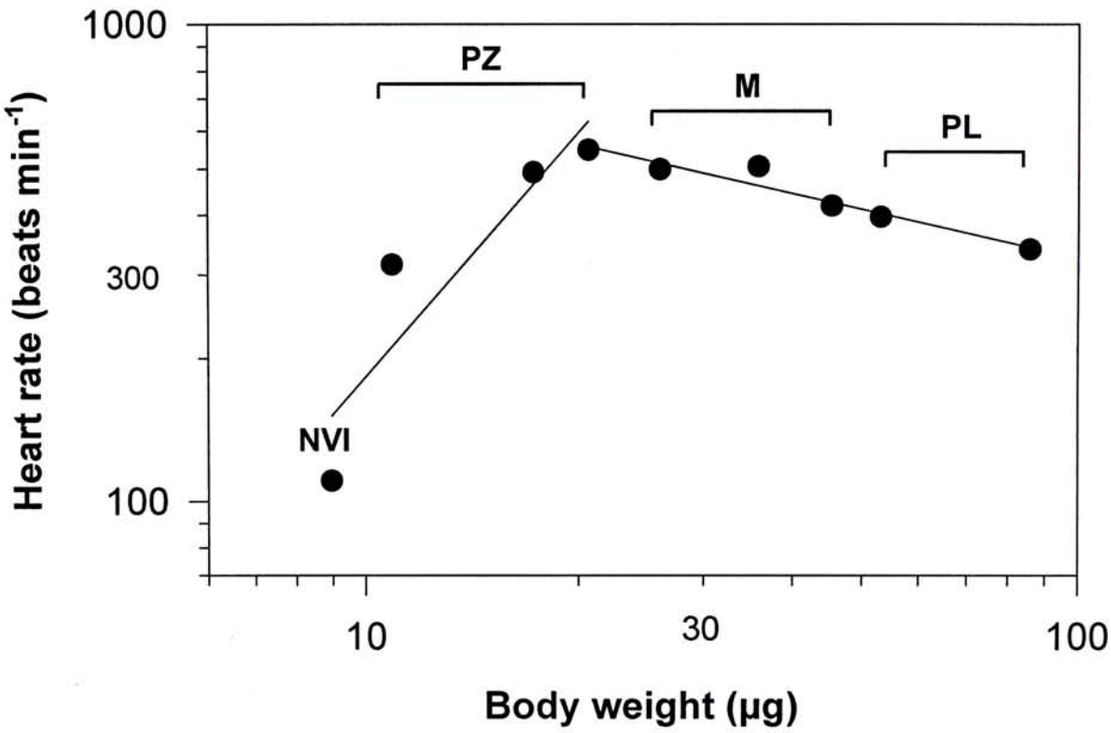



Fig. 3.4 Relationship between heart rate and body weight during development of *Metapenaeus ensis*. NVI: nauplius VI; PZ: protozoea; M: mysis; PL: postlarvae.

lobster. The shrimp nauplii rely on the yolk reserves for nutrition and the shrimp only starts feeding at protozoea I. The lack of cardiovascular functioning in the early nauplii is likely to be associated with the small size of the non-feeding larvae in which oxygen may diffuse readily across the body surface to tissues and nutrient transport is not an issue. When yolk reserves become exhausted in late nauplii (Chu and Ovsianico-Koulikowsky, 1994), the circulatory system would become necessary for maximal gaseous exchange as well as nutrient distribution. In newly hatched lobster and crayfish of larger size, a circulatory system would be necessary upon hatching and development of the heart commences in the embryos.

In *Metapenaeus ensis*, the heart rate increased dramatically from protozoea I and reached a peak at protozoea III but declined gradually from mysis I onwards. A rapid increase in heart rate after the onset of cardiac functioning followed by a decrease in heart rate with continuing development was also reported in the water flea *Daphnia magna*, the brine shrimp *Artemia franciscana*, the amphipod *Gammarus duebeni*, the lobster *Nephrops norvegicus* (Spicer and Morritt, 1996) and the crayfish *Procambarus clarkii* (Wojciechowski and McMahon, in preparation). The study on swimming behavior of *Metapenaeus ensis* larvae showed that protozoeae are continuous swimmers, and there is an increase in swimming speed during protozoeal development, reaching a peak at protozoea III (Chu *et al.*, 1996). High oxygen consumption due to high swimming activity and fast developmental rate (Chu and Ovsianico-Koulikowsky, 1994) may explain why protozea III had the highest heart rate among all stages studied. The swimming speed in subsequent developmental stages is lower and swimming is often interrupted by resting period.

A reduction in activity may contribute to lower oxygen consumption, leading to lower heart rates observed in mysid and postlarval stages. With the information on stroke volume and cardiac output, the results could be more complete. However, in the present study, stroke volume could not be measured accurately due to the complicated geometrical shape of the heart.

In the present study, a biphasic relationship was found between heart rate and body weight during larval development. The biphasic relationship involves a period in which heart rate increases despite increase in body weight, followed by a period which heart rate decreases with increase in body weight. The general relationship of a decrease in heart rate with increased body weight was found by Schwartzkopff (1955) and Maynard (1960b). Schwartzkopff (1955) studied the heart rate as a function of body weight in a variety of crustaceans including both branchiopods and decapods. He demonstrated that the heart rate decreased with the increasing size of the animals. The relationship can be expressed in the formula: $F = a X^{b}$ where F is the heart rate in beats $min^{-1}$, X is the body weight of the animal, $a$ is the constant and $b$ is the regression coefficient. The relationship $F = 160X^{-0.12}$ for the heart rate of a variety of crustaceans to body weight at 20°C was obtained. That is, the heart rate varied according to the -0.12 power of the body weight. A comparison of heart rate data from crustaceans ranging from larvae to adult of widely differing body weight confirmed the general relationship that heart rate decreases with increase in body weight (Maynard, 1960b). The results from the present study showed that the heart rate increased with body weight from nauplius VI to protozoea III but then decreased with body weight from protozoea III to benthic postlarvae. The $b$ value obtained

from nauplius VI to protozoea III indicates that heart rate increased at 1.70 power of body weight. Spicer and Morritt (1996) also demonstrated a biphasic relationship between heart rate and body weight in the brine shrimp *Artemia franciscana*, the water flea *Daphnia magna*, the amphipod *Gammarus duebeni*, and the lobster *Nephrops norvegicus*. The *b* values of the above animals in the first half of biphasic relationship are 1.92 *(A. franciscana)*, 0.71 (*D. magna* clone), 0.76 (wild *D. magna*), 0.7 (*G. duebeni*), and 0.16 *(N. norvegicus)*. All the values suggested an increase in heart rate with body weight. This rapid increase of heart rate from nauplius VI to protozoea III in *M. ensis* can be explained by the rapid development of the heart and the high activity of the animals in these stages. From protozoea III to benthic postlarvae, the value of *b* indicates that heart rate decreased at 0.34 power of body weight. This value is within the range of *b* values found in other crustaceans. Spicer and Morritt (1996) showed that the *b* values in the second half of biphasic relationship of the animals studied are -0.67 (*A. franciscana*), -0.16 (*D. magna* clone), -0.26 (wild *D. magna*), -0.14 (*G. duebeni*), and -0.15 (*N. norvegicus*). The value found by Schwartzkopff (1955) on a variety of crustaceans is -0.12 and that found by Maynard (1960b) in the spiny lobster *Panulirus argus* is -0.11. All the values showed a general relationship that smaller animals had a higher heart rate than large ones. The *b* value found in the present study is -0.34 (standard error = 0.04) which is significantly different ($P<0.005$) from the value of -0.12 found by Schwartzkopff (1955) for a variety of crustaceans. The results suggest that the decrease in heart rate does not depend solely on the increase in body weight and other factors related to development may affect the heart rate. Spicer (1994) suggested that the initial increase of heart rate with body weight in the brine shrimp

was associated with the period of growth of the heart while the period of decrease of heart rate with increasing body weight was associated with the subsequent period of simple heart elongation. However, this reason cannot be extended directly to the decapod crustaceans where incremental increase in the length of heart is absent (McMahon *et al.*, in press).

Developmental constraints may also play certain role in the construction of a functional cardiovascular system. In *Metapenaeus ensis*, it may also be true that time is needed for full development of the heart. This is supported by the change in heart shape during larval development. The heart changed from a kite-shape in the late naupliar and protozoeal instars to a bottom flattened shape at mysid instars, and then to a rectangular shape at postlarvae. The change in the shape of the heart is possibly related to the development of blood vessels and alary ligaments which stretch the heart into a different shape. Thus the change in the shape of the heart may represent different stages of the development of the circulatory system.

Besides the change in heart rate and heart shape, there may also be a change in the control of heart pulsation during development. The myocardium of adult crustaceans is not spontaneously electrically or mechanically active, but rather is driven by the nervous output of a cardiac ganglion (see 2.1.2.1). However, there have been studies suggesting that crustacean hearts are myogenic very early in development and later become neurogenic (Yamagishi, 1990; Yamagishi and Hirose, 1992). In the present study, before the stage of MII or MIII, the shrimp show no response to the vibration from the great hit, suggesting they have a myogenic heart.

The change of heart from myogenic to neurogenic may be related to the development of nervous system. Thus, from MII or MIII instar onwards, the heartbeat of shrimp would stop for a few milliseconds in response to the vibration by application of a great hit on the bench during the heart rate measurement. Similarly, in the study on embryonic heart development in the crayfish *Procambarus clarkii*, a stimulus response was first observed during the 6$^{th}$ cardiac developmental stage as evidenced by a period of cardiac arrest lasting a few milliseconds (Wojciechowski and McMahon, in preparation). This reflex response to vibration suggests the presence of a coordinated neural system capable of perceiving changes in environment. The results indicate that the heart of *P. clarkii* is myogenic before the 6$^{th}$ cardiac developmental stage and later becomes neurogenic. In *Homarus americanus*, the heart begins to pulse rhythmically in 4 to 5 week-old embryos, long before nervous innervation is established (Herrick, 1909). Yamagishi (1990) also suggested that the heart in early juveniles of the isopod *Ligia exotica* is myogenic, but this myogenicity becomes subordinate to neural drive during development. Further studies on anatomical changes on heart innervation during development are required.

# CHAPTER 4

# EFFECT OF SALINITY ON HEART RATE OF *METAPENAEUS ENSIS*

## 4.1 Introduction

Physiological studies of early developmental stages are necessary for an understanding of physiological adaptations of these stages to environmental stress, particularly since these may involve responses quite different from those of the adult. Further, studies on the development of physiological structures and their functions from the larvae to adult are of interest. One of the systems which undergoes major changes during development is the cardiovascular system. To be of adaptive value, a cardiovascular system must supply blood where it is needed, in adequate amount and when required (Farrell, 1991). Several studies indicated that heart rate in adult crustaceans is an indicator of stress in response to various environmental factors such as temperature, oxygen level and pollution. Examples included studies on the crab *Cancer magister* (De Wachter and McMahon, 1996; Airriess and McMahon, 1994) and the water flea *Daphnia* (Gliwicz and Sieniawaska, 1986).

The life cycle of penaeid shrimps involves a migratory pattern between waters of different salinities (Panikkar 1968; Wickins 1976). The ovigerous females spawn in areas of high salinity and the resulting larvae gradually drift towards

regions of low salinity along the shore. The postlarvae enter estuaries where they develop and when they become mature, they return to the sea for breeding. This migratory pattern suggests that the various ontogenetic stages may differ in their physiological responses to osmotic stress. Preston (1985) has reported an increase in low salinity tolerance in the mysis stage of *Metapenaeus bennetae*.

*Metapenaeus ensis*, a commercially important penaeid shrimp in southern China, has a life cycle similar to other penaeid shrimps. Chu and So (1987) demonstrated a progressive increase in tolerance to low salinity during development in *Metapenaeus ensis*. Nauplii cannot survive at a salinity of 15‰. The survival of protozoeae is often lower at 20‰ than at higher salinities. However, myses and postlarvae can withstand a salinity of 15‰.

The objective of the present study is to examine the effect of salinity on the heart rate of the larvae and postlarvae of *Metapenaeus ensis*. This study can be divided into 2 parts. In the first part, the variation in heart rate of shrimp was studied during larval development in salinity of 18‰ and 28‰. The second part examined the reponses of larval shrimp hearts to acute change in salinity. Acute exposure might indicate the inherent ability of the experimental animals to tolerate a sudden salinity change (Knowlton and Schoen, 1984).

## 4. 2 Materials and Methods

The procedure for spawning and larval rearing of *Metapenaeus ensis* for this study has been described in 3.2. To study the effect of salinity on the heart rate of the shrimp during larval development, shrimp from the same spawning were reared in two 10-l aquariums maintained at two different salinities, 28‰ and 18‰, starting from protozoea I (PZI) at a density of about 100 individuals $l^{-1}$. Salinity in one of the aquariums was lowered from 28 to 18‰ in a stepwise fashion by diluting with distilled water to 24‰ and then to 20‰ and finally to 18‰ each at an interval of a few hours. This step was necessary in order to minimize a sudden change in salinity for the young larvae which would affect their development and metamorphosis. The temperature was kept at 28°C by an aquarium heater. Other rearing conditions including food applications have been described in 3.2. The heart rate of shrimp at protozoea III (PZIII), mysis III (MIII), planktonic postlarvae [PL at day 2 to 4, PL(p)] and benthic postlarvae [PL at day 7 to 9, PL(b)] were chosen as representative stages for measurement with micro-videophotography. The procedure for measuring heart rate was the same as described in 3.2.

Since the acclimation time of different stages to 18‰ seawater was different in the above experiment, i.e., older stages were acclimation longer than younger ones, another experiment was done to determine heart rate of the different stages after the same acclimation period at 18‰. In the above experiment, when the heart rate of PZIII was measured, it had been acclimated to 18 ‰ for 3 days. Thus in this series of experiment, for each of the other stages studied, the salinity was lowered to

18‰ in a stepwise fashion only three days before heart rate measurement so that every stage studied was acclimated for the same period of time.

To study the heart rate of shrimp in response to acute salinity exposure, shrimp from the same spawning were reared at 28‰ and 18‰ as described above. The heart rate of shrimp from the same selected stages was measured using micro-videophotography in continuous perfusion experiments. The experimental setup is shown in Fig. 4.1 (McMahon *et al.*, 1995). Two syringes containing 18‰ and 28‰ seawater respectively were set in the syringe pumps (Temuro model STC-521 and 523). The seawater used in the test was well aerated by bubbling and was filtered through 0.45 μm mesh to prevent the blockage of the system. Shrimp were placed in a chamber constructed from a 25 μl pipette which could be continually perfused with seawater using the syringe pumps. Fine stainless steel rods were placed at either ends of the pipette. Their positions could be controlled by magnets so that the shrimp could be kept in position under the microscope and camera. The flow rate was adjusted to 1 ml $h^{-1}$ to maintain the oxygen level but without disturbing the shrimp. After placing the shrimp into the chamber, shrimp reared at 28‰ were perfused with 28‰ seawater for 20 minutes for acclimation. Measurements were made at 10-min interval in this acclimation period. Then, 18‰ seawater was delivered for 25 minutes and measurements were made at the $0^{th}$, the $10^{th}$ and the $15^{th}$ min of the test period. After the test, 28‰ seawater was delivered for 20 min to allow recovery. In the recovery period, measurement was made at 10 min interval. The procedures were identical when exposing shrimp reared at 18‰ seawater to 28‰ seawater except that the shrimp were reared at 18‰ so that 18‰ seawater was

Figure 4.1 Experimental setup for measurement of heart rate in response to salinity variation.

perfused at acclimation and recovery period while 28‰ seawater was perfused at test period.

## 4.3 Results

The heart rates of shrimp during larval development in salinity of 18‰ and 28‰ are shown in Fig. 4.2. Heart rate of shrimp measured under different salinity regimes were compared by Student's t-test or One-way ANOVA. Results showed that the heart rate of MIII, PL(p) and PL(b) reared at 18‰ was not significantly different from those reared at 18‰ for 3 days (One-way ANOVA, $P>0.05$). The heart rate of PZIII reared at 18‰ was about 20% significantly higher than those reared at 28‰ (Student's t-test, $P<0.05$). The heart rate of MIII and PL(p) reared at 18‰ and those reared at 18‰ for 3 days was about 10 to 20% significantly higher than those reared at 28‰ (One-way ANOVA followed by Student-Newman-Keuls test, $P<0.05$). The heart rate of PL(b) reared at 18‰ or 18‰ (3 days) was not significantly different from those reared at 28‰ (One-way ANOVA, $P>0.05$). These results implied that the difference between heart rate of shrimp reared at the two salinities was related to different developmental stage but not different acclimation period.

The heart rates of shrimp reared at 28‰ and exposed to acute salinity change to 18‰ seawater are shown in Fig. 4.3. Heart rates at different measurement periods

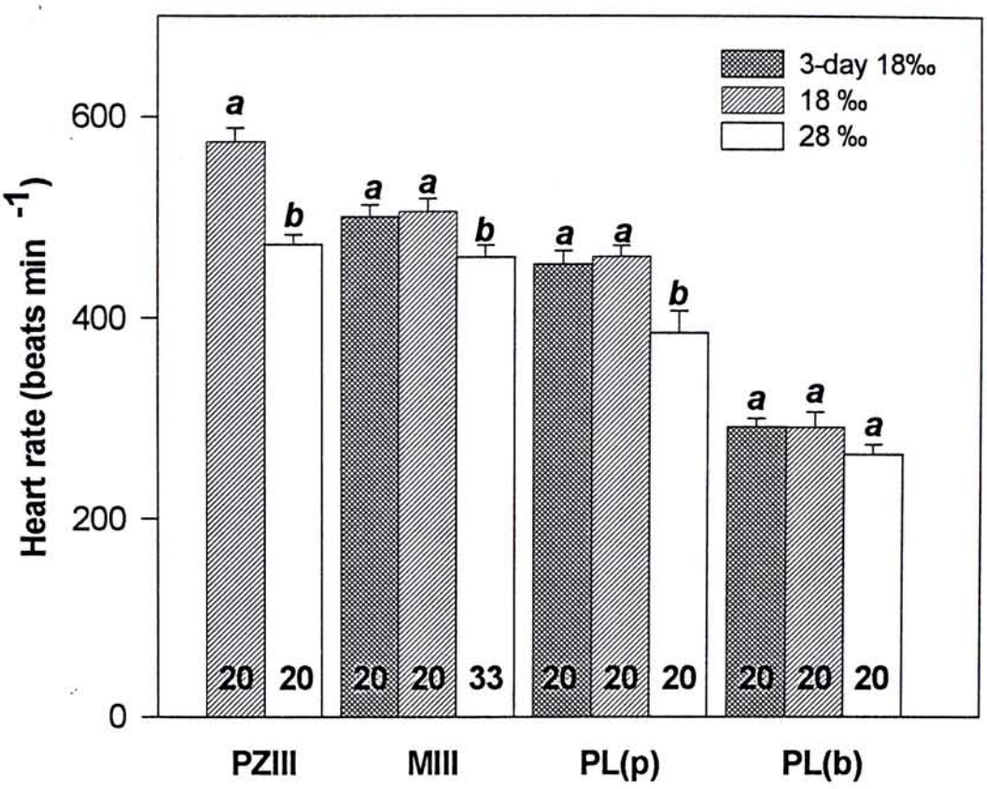



Fig. 4.2 Heart rate in selected ontogenetic stages of *Metapenaeus ensis* reared at 18‰ seawater and 28‰ seawater. PZIII: protozoea III; MIII: mysis III; PL(p): planktonic postlarvae; PL(b): benthic postlarvae. Crossed bars represent shrimp reared at 18‰ for 3 days. Striped bars represent shrimp reared at 18‰. Open bars represent shrimp reared at 28‰. Values are means ± standard error. The numbers inside the bar indicated the sample size. Values with different letters in the same group of bars are significantly different [$p<0.05$; Student's t-test for PZIII and One-way ANOVA, followed by Student-Newman-Keuls test for MIII, PL(p) and PL(b)].

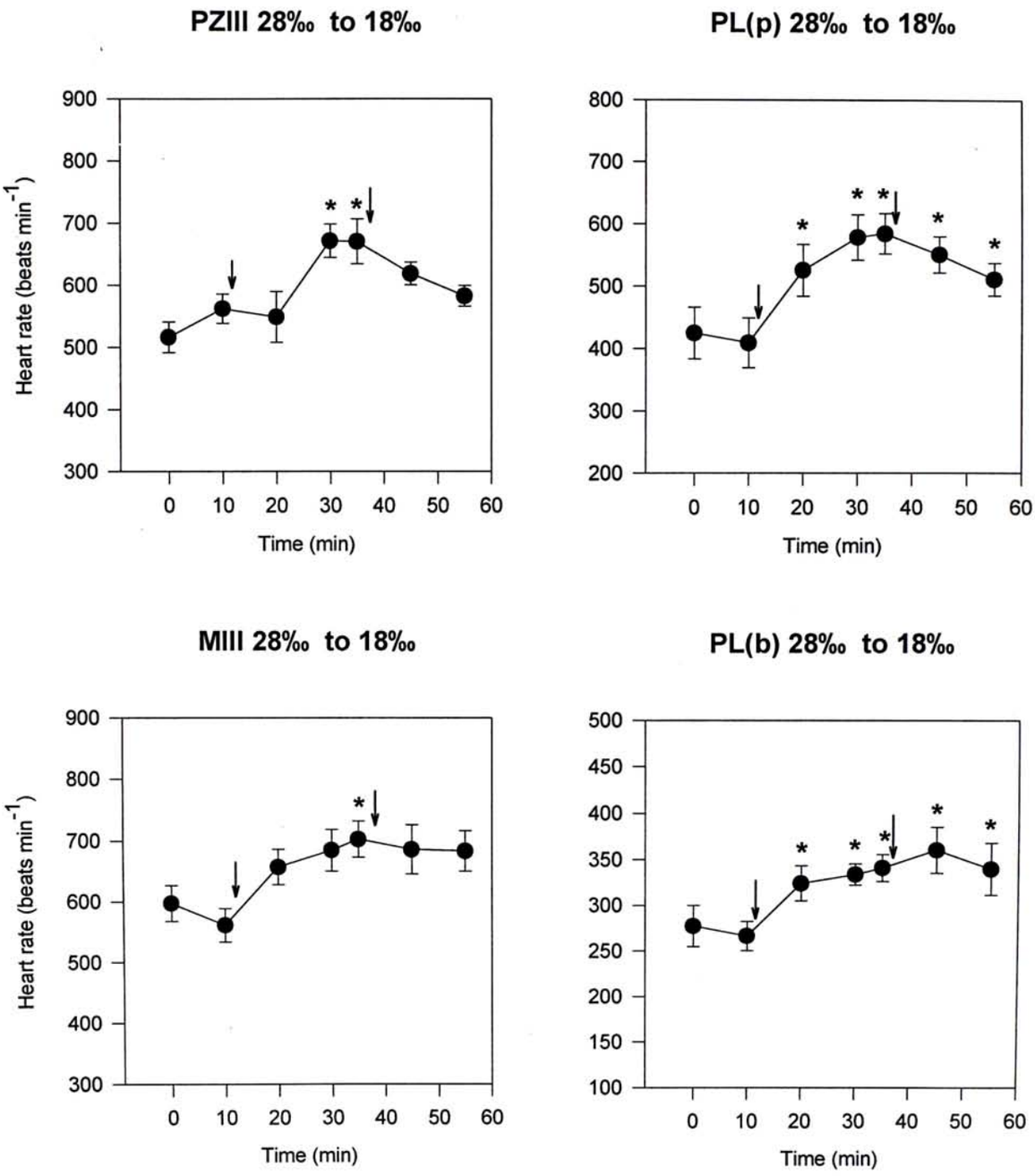



Fig. 4.3 Change in heart rate of selected ontogenetic stages of *Metapenaeus ensis* reared at 28‰ seawater in response to acute salinity exposure to 18‰ seawater. PZIII: protozoea III; MIII: mysis III; PL(p): planktonic postlarvae; PL(b): benthic postlarvae. Values are means ± standard error, n = 10. The arrows indicate when the salinity was changed. Asterisks denote significant differences between the heart rate concerned and the heart rate measured at time = 0 ($p < 0.05$; One-way ANOVA with repeated measures, followed by Student-Newmans-Keuls test).

were analyzed by One-way ANOVA with repeated measures, followed by Student-Newman-Keuls test. Results showed that when PZIII, MIII, PL(p) and PL(b) reared at 28‰ were exposed to 18‰ seawater, they showed about 20 to 35% increase in heart rate during the test ($P<0.05$). In the recovery period, the heart rate of PZIII and MIII returned to the pre-test level ($P>0.05$) while the heart rate of the other stages did not ($P<0.05$).

The heart rates of shrimp reared at 18‰ and exposed to 28‰ seawater are shown in Fig. 4.4. Results showed that when PZIII, PL(p) and PL(b) reared at 28‰ were exposed to 18‰ seawater, there were no significant changes in heart rate (One-way ANOVA with repeated measures, $P>0.05$). Yet, the heart rate of MIII reared at 28‰ significantly decreased by about 20% when animals were exposed to 18‰ seawater (One-way ANOVA with repeated measures, followed by Student-Newman-Keuls test, $P<0.05$). In the recovery period, the heart rates of MIII returned to pre-test level ($P>0.05$).

## 4.4 Discussion

Different developmental stages encounter different environmental regimes, with physiological structures especially adapting to the respective environmental regimes. Physiological responses of early developmental stages possibly is quite different from those of adults. The ability of salinity tolerance and osmotic regulation in different life history stages can be correlated with their distribution

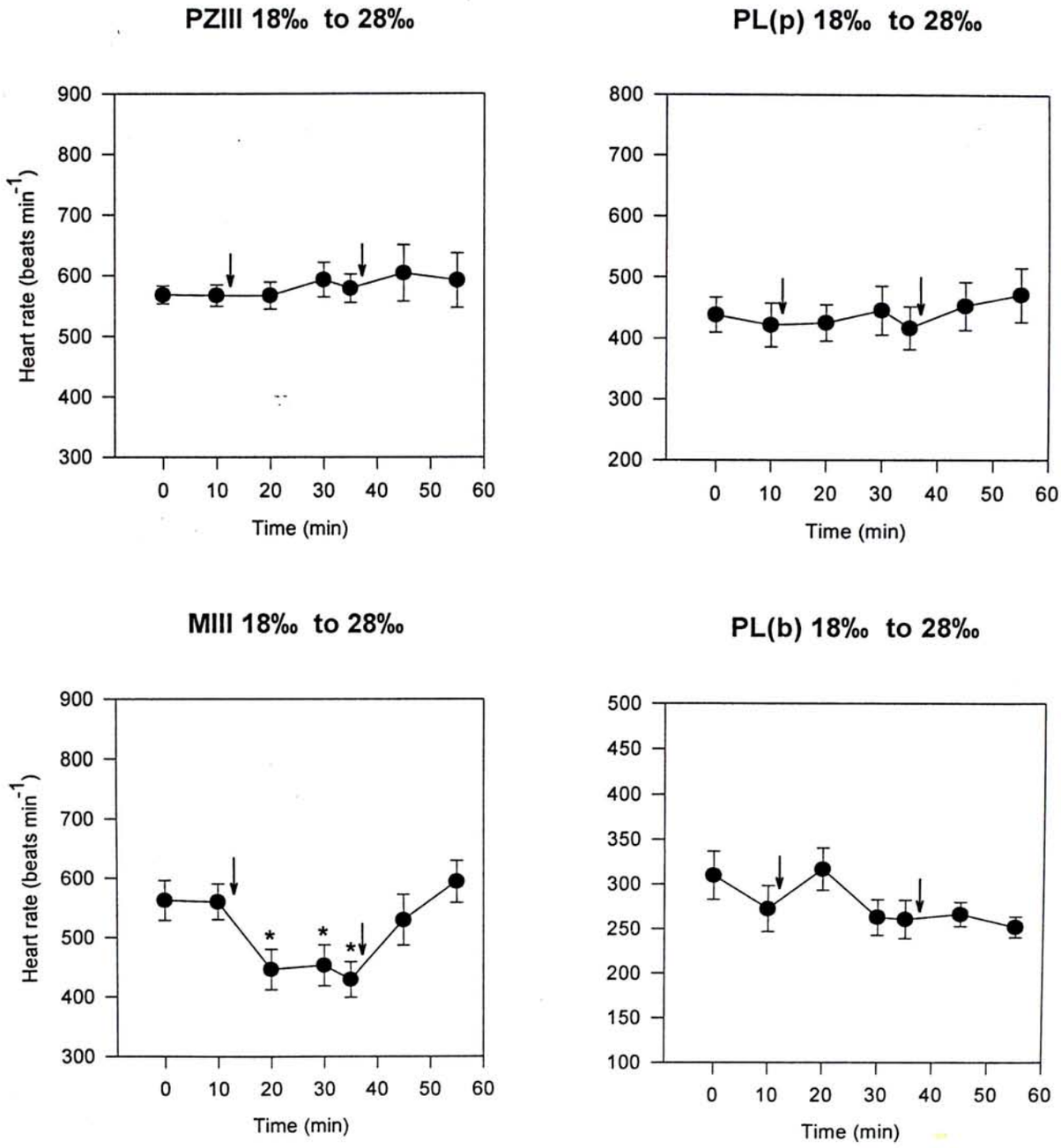



Fig. 4.4 Change in heart rate of selected ontogenetic stages of *Metapenaeus ensis* reared at 18‰ seawater in response to acute salinity exposure to 28‰ seawater. PZIII: protozoea III; MIII: mysis III; PL(p): planktonic postlarvae; PL(b): benthic postlarvae. Values are means ± standard error, n = 10. The arrows indicate when the salinity was changed. Asterisks denote significant differences between the heart rate concerned and the heart rate measured at time = 0 ($p < 0.05$; One-way ANOVA with repeated measures, followed by Student-Newmans-Keuls test).

pattern in the natural environment. The life cycle of penaeid shrimp involves migration between high and low salinities (Panikkar 1968; Wickins 1976). The ovigerous females spawn in areas of high salinity and the resulting larvae gradually drift towards region of lower salinity along the shore. The postlarvae enter estuaries where salinity is highly fluctuated. This correlates with the increase in salinity tolerance and osmoregulatory ability seen as the penaeid shrimp develop. *Metapenaeus ensis* presumably with a life cycle similar to other penaeid shrimps and therefore would also exhibits an increase in salinity tolerance and osmoregulatory ability during development (Chu and So, 1987). The results from the present study showed that the heart rate of protozoea III, mysis III and planktonic postlarvae reared at 18‰ was higher than those reared at 28‰. This can be associated with the fact that larvae and early postlarvae inhabit areas of high salinity; a salinity of 18‰ which is outside their preferred salinity range is thus stressful to them. Study of the shrimp *Penaeus japonicus* showed that ATPase activity is absent in nauplii and protozoea I suggesting that these stages are probably osmoconformers and are not tolerant to low salinities (Bouaricha *et al.*, 1994). In mysis II and III, osmoregulatory tissues are present in the branchial chambers, on the inner side of the branchiostegites, and on the pleutae. The gills and the first epipodites appear as buds at these stages. The activity of $Na^+$-$K^+$ ATPase increases slightly but these stages retain an osmoconforming type of regulation and have a low tolerance to variations in salinity. In postlarvae, osmoregulatory tissues appear in the gills and mainly in the epipodites. ATPase activity increases and reaches maximum in benthic postlarvae. Assuming that the development of osmoregulatory structures of *Metapenaeus ensis* is similar to *Penaeus japonicus*, protozoea III and mysis III of

*Metapenaeus ensis* are probably also osmoconformers while the osmoregulatory ability of planktonic postlarvae is not yet fully developed. Therefore, a salinity of 18‰ is stressful to them and leads to an increase in heart rate. In response to salinity stress, crustaceans exhibit physiological and behavioral responses. Previous studies have shown that oxygen consumption increases when crustaceans were exposed to low salinity. Ting (1970) reported that a decrease of salinity from 40 to 10‰ resulted in an increase in oxygen consumption of both *Penaeus monodon* and *Metapenaeus monoceros*. Direct transfer of larvae of *Callinectes sapidus* to reduced salinity water induced a large increase in metabolism (Leffler, 1975). Increased oxygen consumption has also been reported in the adult penaeid shrimp *Penaeus monodon* and *P. stylirostris* under low salinity stress (Gaudy and Sloane, 1981). Dalla Via (1986) reported that oxygen consumption of *Penaeus japonicus* increased rapidly to 300% of the initial value and stabilized at 200% after a few hours with a change in salinity from 37 to 10‰. *Homarus americanus* showed avoidance response when exposed to low salinity (Jury *et al.*, 1994b). The increase in oxygen consumption imposed by salinity stress can be adjusted by cardiovascular system via change in cardiac output, heart rate, stroke volume and hemolymph flow distribution. In the present study, stroke volume could not be measured accurately due to the complicated geometrical shape of the heart. Assuming there is little change in stroke volume, the observed increase in heart rate would result in an increase in cardiac output. Jury *et al.* (1994b) had reported that there is a close relationship between oxygen consumption and heart rates. Besides the increase in oxygen consumption, the increase in heart rate during exposure to low salinity may also be due to a change in the ionic composition of hemolymph reaching the heart, a change in blood volume

and the release of hormones. Several pericardial hormones such as 5-hydroxytryptamine have been shown to modulate heart rate and alter hemolymph flow in *Cancer magister* (Airriess and McMahon, 1992; McGaw *et al.*, 1994). Therefore, a higher heart rate is required to give a higher cardiac output in order to meet the higher oxygen demand of protozoea III, mysis III and planktonic postlarvae with osmoregulatory ability not yet fully developed.

The results of the acute exposure experiments in this study suggest that the elevated heart rate under low salinity stress was induced very rapidly upon a change in salinity. In all stages, when shrimp reared at 28‰ were exposed to 18‰, heart rate increased. Heart rate in protozoea III and mysis III returned to initial level after return to 28‰ for 20 minutes but the heart rate of postlarvae did not. Apparently, postlarvae need a longer period for recovery of heart rate. The long time for recovery may be due to a long lasting hormonal effect occurred during exposure to low salinity which altered heart rate. The sudden drop in salinity from 28‰ to 18‰ is stressful to shrimp in all stages. The shrimp probably escapes, leading to an increase in oxygen consumption. The instant response of heart rate to stress may allow the use of heart rate as a bioindicator of environment pollution and other stress. Further studies are required to develop the use of heart rate as bioindicator.

The heart rate of benthic postlarvae reared at 18‰ was not significantly different from those reared at 28‰. The natural habitat of benthic postlarvae are areas of low salinity. Bouaricha *et al.* (1994) showed that ATPase activity increases and reaches maximum in *Penaeus japonicus* benthic postlarvae. Similarly, the

benthic postlarvae of *Metapenaeus ensis* would be better able to compensate for low salinity than the earlier developmental stages. The heart rate was not involved in adaptation to salinity change. However, acute exposure of benthic postlarvae reared at 28‰ to 18‰ seawater did result in an increase in heart rate. This change appears to be only temporary as heart rate of animals acclimated at the two salinities is similar. The shrimp probably tries to escape and thus has increased oxygen consumption due to activities. The short-term change in heart rate may result from a transient change in oxygen consumption in response to salinity stress. The heart rate did not return to the level before test in 20 min. Apparently, the length of recovery period is too short.

The heart rate of shrimp reared at 18‰ did not change on exposure to 28‰ for all stages studied, except in mysis III where a decrease in heart rate was observed. No increase in heart rate was ever recorded. The results suggested that the elevated heart rates which develop in acclimation to low salinity conditions do not respond readily to an acute increase in salinity. In support of the former conclusion, Dalla Via (1987) reported that oxygen consumption is the lowest at the iso-osmotic point in *Palaemonetes antennarius*. As the iso-osmotic point for penaeids ranges from 24 to 29‰ (Parado-Estepa *et al.*, 1987), the transition to 28‰ which is close to the iso-osmotic point of the shrimp would not lead to an increase in oxygen consumption. Thus an increase in heart rate is not necessary. Therefore, this transition may not be drastic enough to elicit a heart rate response or the responses would develop after a longer period of time.

In the present study, only the response of heart rate to salinity change was studied. Studies on other cardiovascular functions, particularly the change in stroke volume and other physiological parameters including changes in oxygen consumption and osmotic concentration in the hemolymph are needed for a comprehensive understanding on the cardiovascular adjustment to low salinity. For instance, McGaw and McMahon (1996) showed that the increase in heart rate recorded during exposure of *Carcinus maenas* to low salinity did not lead to an increased cardiac output. Instead, an overall decreased cardiac output resulted from a substantial decrease in heart stroke volume. When similar information on *Metapenaeus ensis* becomes available, a more complete understanding of the response of its cardiovascular functions to variation in salinity can be obtained.

# CHAPTER 5

# EFFECT OF TEMPERATURE ON HEART RATE OF *METAPENAEUS ENSIS*

## 5.1 Introduction

Temperature limits the distribution of living organisms and is an important determinant of their activities. Maintenance of effective organismic integrity by regulating the balance between the rates of various biochemical activities is very important for survival. The study on the temporal relationships between environmental physico-chemical conditions and heart rate in *Carcinus maenas* showed that temperature is the principal factor that influences the heart rate compared with light intensity, salinity and depth (Aagaard, 1996). This is because *Carcinus maenas* is a poikilotherm and its metabolic rate is proportional to temperature. Heart rate has been shown to increase with temperature in a variety of crustaceans, such as the shore crab *Cancinus maenas* (Ahsanullah and Newell, 1971; Aagaard, 1996), Dungeness crab *Cancer magister* (McMahon *et al.*, 1978; De Wachter and McMahon, 1996), blue crab *Callinectes sapidus* (Burton *et al.*, 1980), crab *Hemigrapsus sanguineus* (Depledge, 1984), crayfish *Cherax tenuimanus* (Villareal, 1990) and squat lobster *Munida rugosa* and *Munida sarsi* (Zainal *et al.*, 1992). The effect of temperature on the adult crustacean circulatory system is thus relatively well studied. However, the effect of temperature on larval crustacean

circulatory system is poorly understood. The objective of the present study is to examine the heart rate of the larvae and postlarvae of the shrimp *Metapenaeus ensis* raised at 25°C in response to acute decrease (20°C) and increase (30°C) in ambient temperature. Temperature coefficient ($Q_{10}$) which reflects the effect of temperature on reaction rates was determined.

## 5.2 Materials and Methods

The procedures for spawning and larval rearing of *Metapenaeus ensis* for this study have been described in 3.2 except that in this case the shrimp studied were maintained at 25±1°C. To study the heart rate of shrimp in response to acute variation in temperature, protozoea III (PZIII), mysis III (MIII), planktonic postlarvae [PL day 2 to 4: PL(p)] and benthic postlarvae [PL day 7 to 9: PL(b)] were chosen as the representative stages for measurement with micro-videophotography. The setup for micro-videophotography was the same as described in 3.2 except that the microscope (Nikon SE) was connected to the video camera (Teli CCD color camera CS5110) and the video signal was directly recorded on a video TV (Sharp VT-1418M). The setup of the chamber containing the shrimp was the same as described in 4.2 except that the pipette which contained the shrimp was surrounded by a water jacket and perfused with 25±1°C seawater. The temperature of the water jacket was controlled by a temperature controller (CAMBION 132) and monitored by a thermocouple. In this way, the ambient temperature of the shrimp could be adjusted instantly. The experimental setup is shown in Fig. 5.1.

Figure 5.1 Experimental setup for measurement of heart rate in response to temperature variation.

In a series of experiments, the chamber containing the shrimp was perfused with 25°C seawater for 30 minutes for acclimation. After this acclimation period, heart rate at 25°C was measured at 5-min interval for 20 min. Then the shrimp was exposed to 20°C by changing the temperature of the water jacket from 25 to 20°C over a 5-min period. The 20°C test period lasted for 20 min and measurement was made at 5-min interval throughout the test period. After the test, temperature was changed back to 25°C within 5 min. Seawater at 25°C was passed through the chamber for 45 min to monitor the responses during the recovery. In the recovery period, measurement was made at the $5^{th}$, $10^{th}$, $15^{th}$, $25^{th}$, $35^{th}$ and $45^{th}$ min. The shrimp was then exposed to 30°C by changing the temperature of the water jacket from 25 to 30°C over 5 min. The 30°C test period also lasted for 20 min and measurement was made at 5-min interval during the test period. After the test, the temperature was changed back to 25°C within 5 min. Seawater at 25°C was passed through the chamber for 45 min, allowing the shrimp to recover. In the recovery period, measurement of heart rate was made at the $5^{th}$, $10^{th}$, $15^{th}$, $25^{th}$, $35^{th}$ and $45^{th}$ min. Five animals of each selected developmental stage were tested in this series of experiments. A separate series of experiments was carried out to examine whether the heart rate of the shrimp responded differently when subject to acute exposure to 30°C before exposure to 20°C. The experimental procedures were identical to the one stated above except that shrimp were exposed to 30°C first.

In order to examine whether the change in heart rate measured during the test period represented a transient response or a maintained response, the length of test period of additional experiments on PZIII and PL(b) were extended to 3 hours and

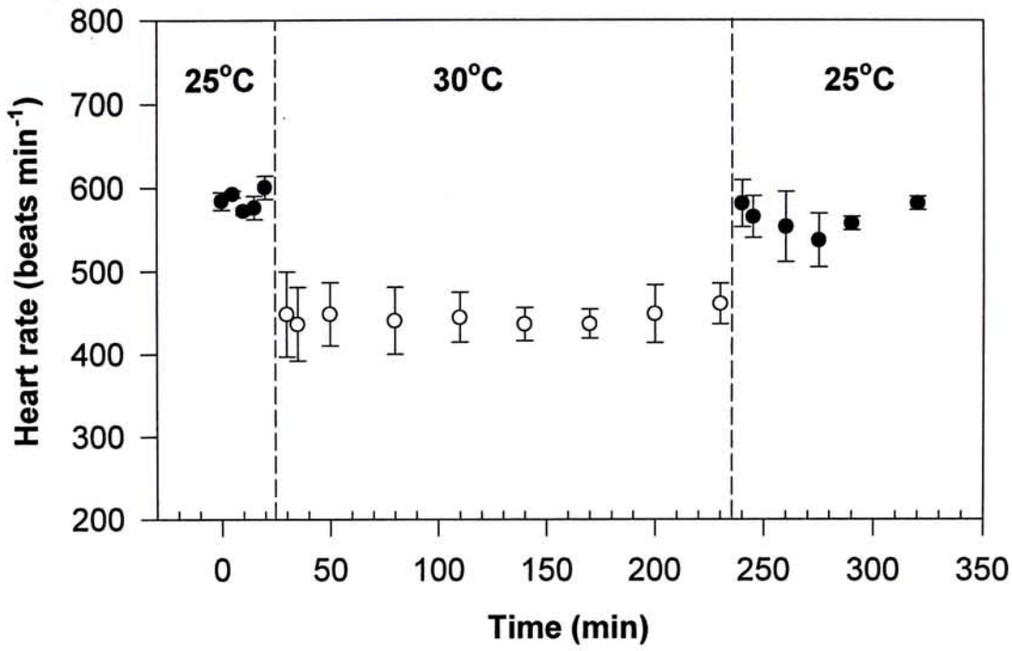
30 min. PZIII was subjected to acute exposure to 30°C while PL(b) was subjected to acute exposure to 20°C. In experiment on PZIII, seawater at 25°C was passed through the chamber containing the shrimp for 30 minutes, allowing the shrimp to acclimate. After that, heart rate at 25°C was measured at 5-min interval for 20 min. Then the shrimp was exposed to 30°C by changing the temperature of the water jacket from 25 to 20°C within 5 min. The 30°C test period lasted for 3 hours and 30 min. In the test period, the first two measurements were made at 5-min interval while the following seven measurements were made at 30-min interval. After the test, the temperature was changed back to 25°C within 5 min. Seawater at 25°C was passed through the chamber for 85 min, allowing the shrimp to recover. In the recovery period, measurement was made at the $5^{th}$, $10^{th}$, $25^{th}$, $40^{th}$, $55^{th}$ and $85^{th}$ min. Three animals of PZIII were tested in this series of experiments. The procedures of the experiment on PL(b) was identical to the one described above except that the temperature of the water jacket was changed to 20°C instead of 30°C during the test period.

## 5.3 Results

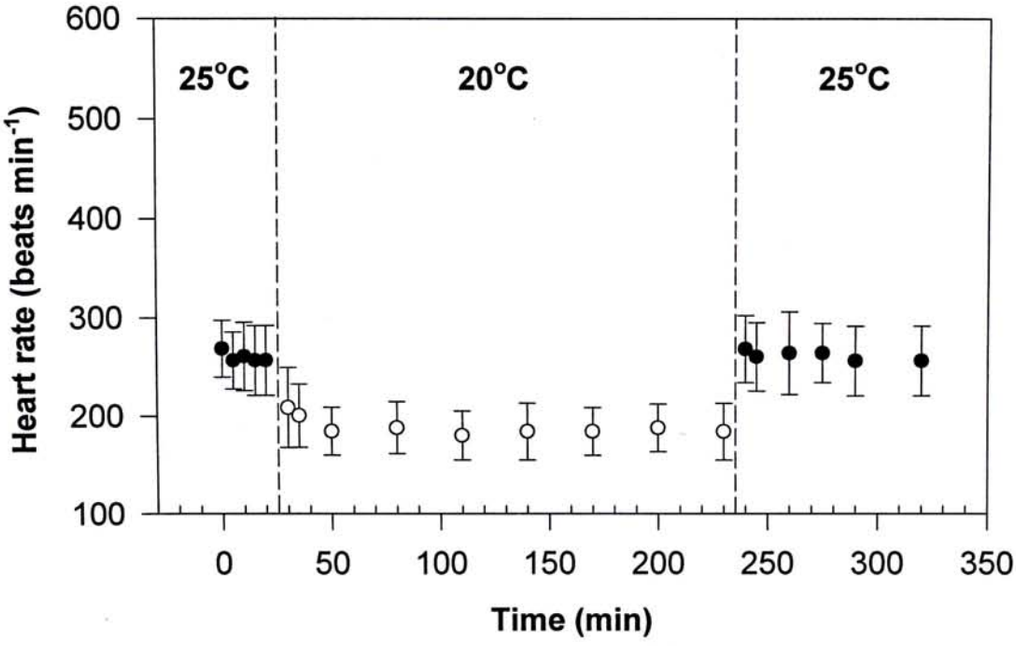
The heart rates measured in an extended test period experiment are shown in Fig. 5.2. The heart rates within acclimation period, test period and recovery period were first independently analyzed by One-way ANOVA. The results showed that there was no significant difference in the heart rate within each period ($P>0.05$). Since there was no variation in heart rate within the 3 hours and 30 min test period,

Fig. 5.2 [a] Change in heart rate of PZIII (protozoea III) reared at 25°C in response to acute exposure to 30°C for 3 hours and 30 min. [b] Change in heart rate of PL(b) (benthic postlarvae) reared at 25°C in response to acute exposure to 20°C for 3 hours and 30 min. Values are means ± standard error, n = 3. Dotted lines indicate when the temperature changed. Values within each temperature period are not significantly different from each other ($p>0.05$). Values of closed circles are not significantly different from the values at the acclimation period ($p>0.05$) and values of open circles are significantly different from the values at the acclimation period ($p<0.05$; One-way ANOVA with repeated measures, followed by Student-Newmans-Keuls test).

## a. PZIII (extended experiment)



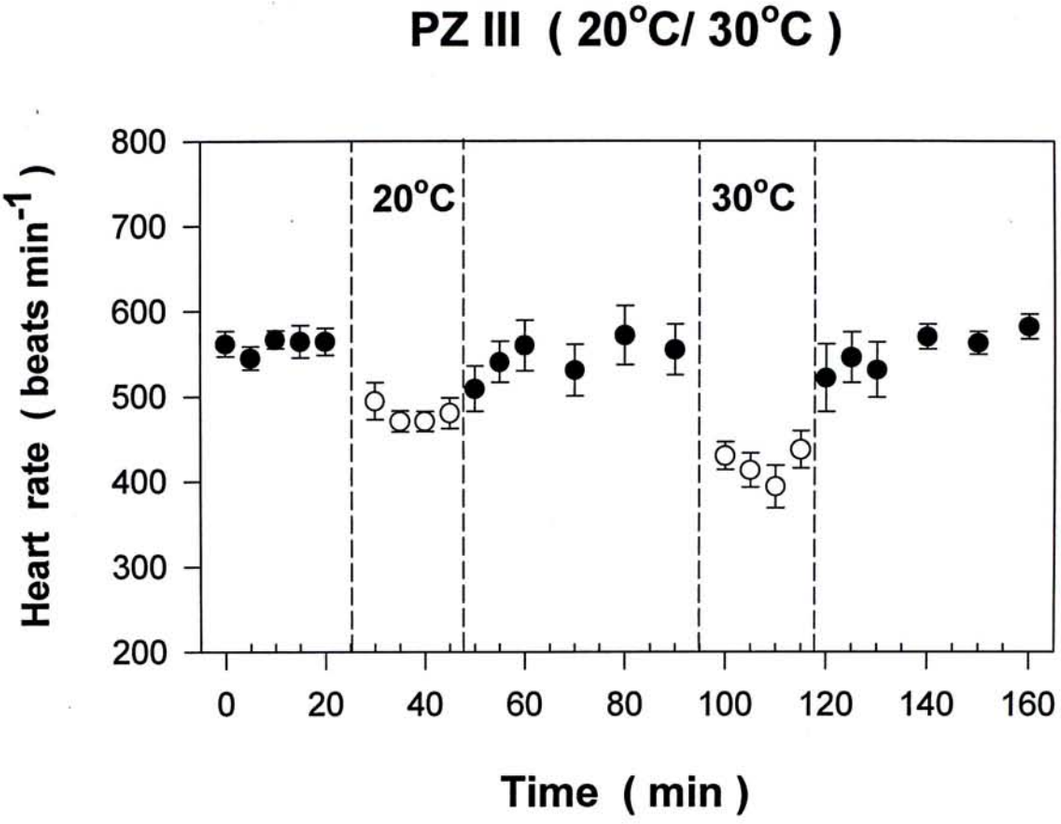

## b. PL(b) (extended experiment)

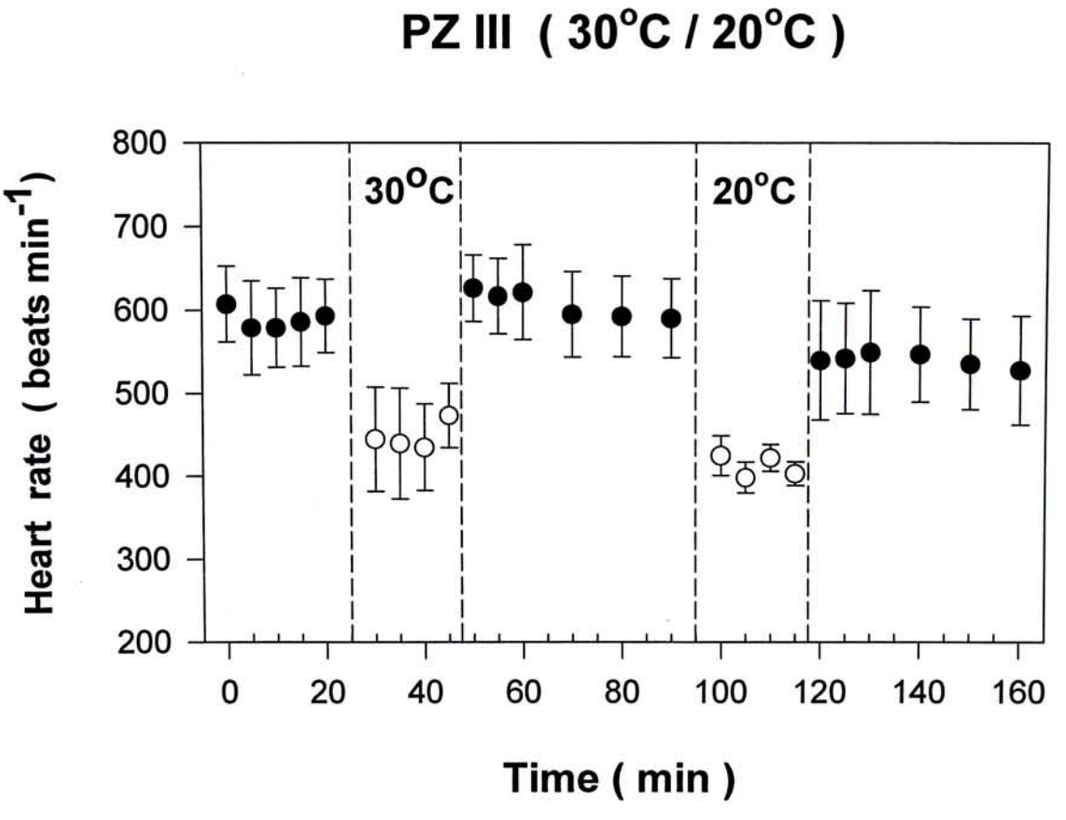


the change in heart rate after a temperature change within 20 min was not a transient response but represented a maintained response. The heart rates measured within each period were then pooled and analyzed by One-way ANOVA followed by Student-Newman-Keuls test. Results showed that the heart rate of PZIII decreased when exposed to 30°C ($P<0.05$) (Fig. 5.2a) and the heart rate of PL(b) decreased significantly when exposed to 20°C ($P<0.05$) (Fig. 5.2b).

Results of the series of experiment which shrimp were exposed to 20°C and 30°C for 20 min are shown in Figs. 5.3 to 5.6. The heart rates of shrimp recorded during different period but within the same temperature were analyzed by One-way ANOVA. Results showed that the heart rates measured in the same period were not significantly different from each other ($P>0.05$). Then, heart rates from the same period were pooled and analyzed by One-way ANOVA with repeated measures followed by Student-Newman-Keuls test. In all four developmental stages except mysis III exposed to 30°C first, the heart rate of acclimation period was not significantly different from the heart rate of the two recovery periods ($P>0.05$) which showed that the heart rate returned to the pre-test level after acute exposure to 20°C and 30°C. The heart rate of the final recovery period was slightly lower ($P<0.05$) than the heart rate of acclimation period in mysis III which exposed to 30°C first. Heart rate of PZIII decreased when exposed to 20°C or 30°C ($P<0.05$) regardless of whether the shrimp were exposed to 20°C or 30°C first (Fig. 5.3a,b). When MIII was exposed to 20°C first, the heart rate decreased in response to 20°C ($P<0.05$). Yet the heart rate of shrimp exposed to 30°C was not significantly different from that measured at 25°C ($P>0.05$) (Fig. 5.4a). However, when MIII was exposed to

Fig. 5.3 [a] Change in heart rate of PZIII (protozoea III) reared at 25°C in response to acute exposure to 20°C first and then 30°C. [b] Change in heart rate of PZIII (protozoea III) reared at 25°C in response to acute exposure to 30°C first and then 20°C. Values are means ± standard error, n = 5. Dotted lines indicate when the temperature changed. Values within each temperature period are not significantly different from each other ($p>0.05$). Values of closed circles are not significantly different from the values at the acclimation period ($p>0.05$) and values of open circles are significantly different from the values at the acclimation period ($p<0.05$; One-way ANOVA with repeated measures, followed by Student-Newmans-Keuls test).


PZ III ( 20°C/ 30°C )
Heart rate ( beats min-1 )
800
700
600
500
400
300
200
20°C
30°C
0
20
40
60
80
100
120
140
160
Time ( min )

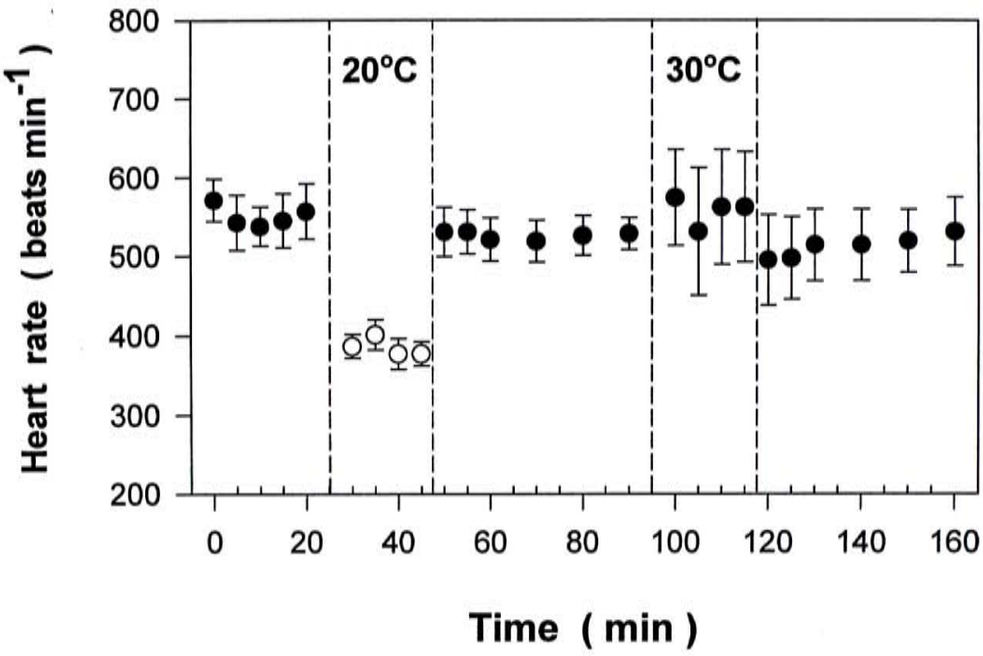

PZ III ( 30°C / 20°C )
Heart rate ( beats min-1 )
800
700
600
500
400
300
200
30°C
20°C
0
20
40
60
80
100
120
140
160
Time ( min )

30°C first, the heart rate decreased in response to 20°C as well as to 30°C ($P<0.05$) (Fig. 5.4b). In both PL(p) (Fig. 5.5a,b) and PL(b) (Fig. 5.6a,b), heart rate decreased when exposed to 20°C ($P<0.05$) and increased when exposed to 30°C ($P<0.05$) regardless of whether the shrimp were exposed to 20°C or 30°C first.

Temperature coefficient ($Q_{10}$) at each developmental stage studied was calculated by the formula: $Q_{10} = (k_2/k_1)^{10/(T°_2-T°_1)}$ where $k_2$ and $k_1$ are the velocity constants at temperatures $T°_2$ and $T°_1$ respectively. The $Q_{10}$ values for PZIII, MIII, PL(p) and PL(b) between 20 and 25°C and the $Q_{10}$ values between 25 and 30°C in experiments with shrimp exposed to 20°C first or exposed to 30°C first were calculated by the above formula (Table 5.1). Results showed that the $Q_{10}$ values calculated from heart rate obtained in experiments with shrimp exposed to 20°C first was not significantly different from that obtained in experiment with shrimp exposed to 30°C first in all developmental stages, except in PZIII (Student's t-test, $P<0.05$). The $Q_{10}$ values of MIII, PL(p) and PL(b) calculated from heart rate in both series of experiments were thus pooled together for further analysis. The $Q_{10}$ values of PZIII and MIII between 20 and 25°C were significantly higher than the corresponding values measured between 25 and 30°C (Student's t-test, $P<0.05$) (Table 5.2). The $Q_{10}$ values of PL(p) between 20 and 25°C were not significantly different from that between 25 and 30°C (Student's t-test, $P>0.05$). However, the $Q_{10}$ values of PL(b) between 20 and 25°C were significantly lower than the corresponding values measured between 25 and 30°C (Student's t-test, $P<0.05$). The $Q_{10}$ values of PZIII, MIII, PL(p) and PL(b) between 20 to 30°C were also calculated and the values increased from 0.98±0.07 ($n = 10$) in PZIII to 2.29±0.13 ($n = 10$) in PL(b) (One-way

Fig. 5.4 [a] Change in heart rate of MIII (mysis III) reared at 25°C in response to acute exposure to 20°C first and then 30°C. [b] Change in heart rate of MIII (mysis III) reared at 25°C in response to acute exposure to 30°C first and then 20°C. Values are means ± standard error, n = 5. Dotted lines indicate when the temperature changed. Values within each temperature period are not significantly different from each other ($p>0.05$). Values of closed circles are not significantly different from the values at the acclimation period ($p>0.05$) and values of open circles are significantly different from the values at the acclimation period ($p<0.05$; One-way ANOVA with repeated measures, followed by Student-Newmans-Keuls test).

a. M III ( 20°C / 30°C )




b. M III ( 30°C / 20°C )

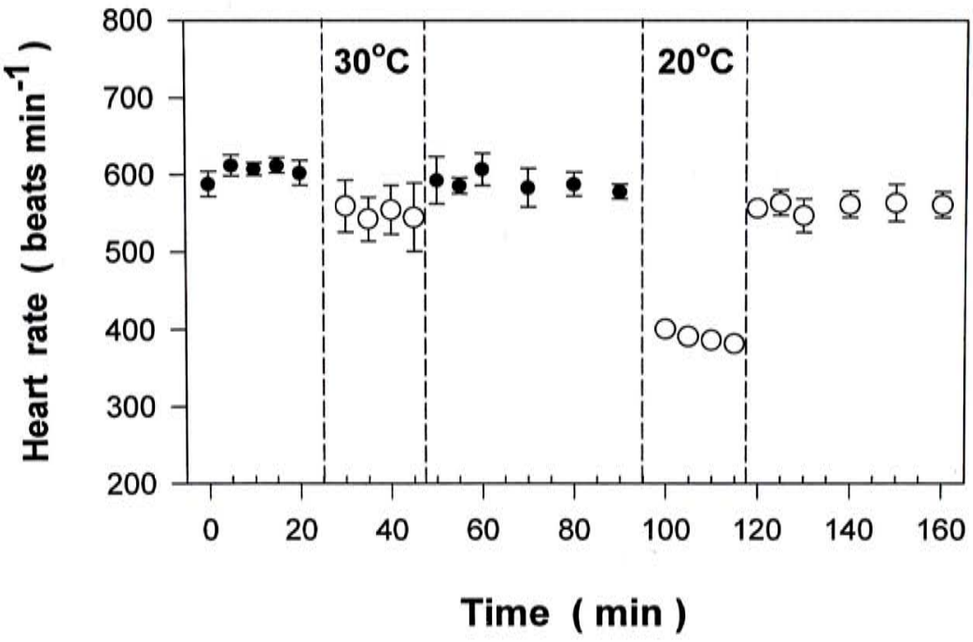
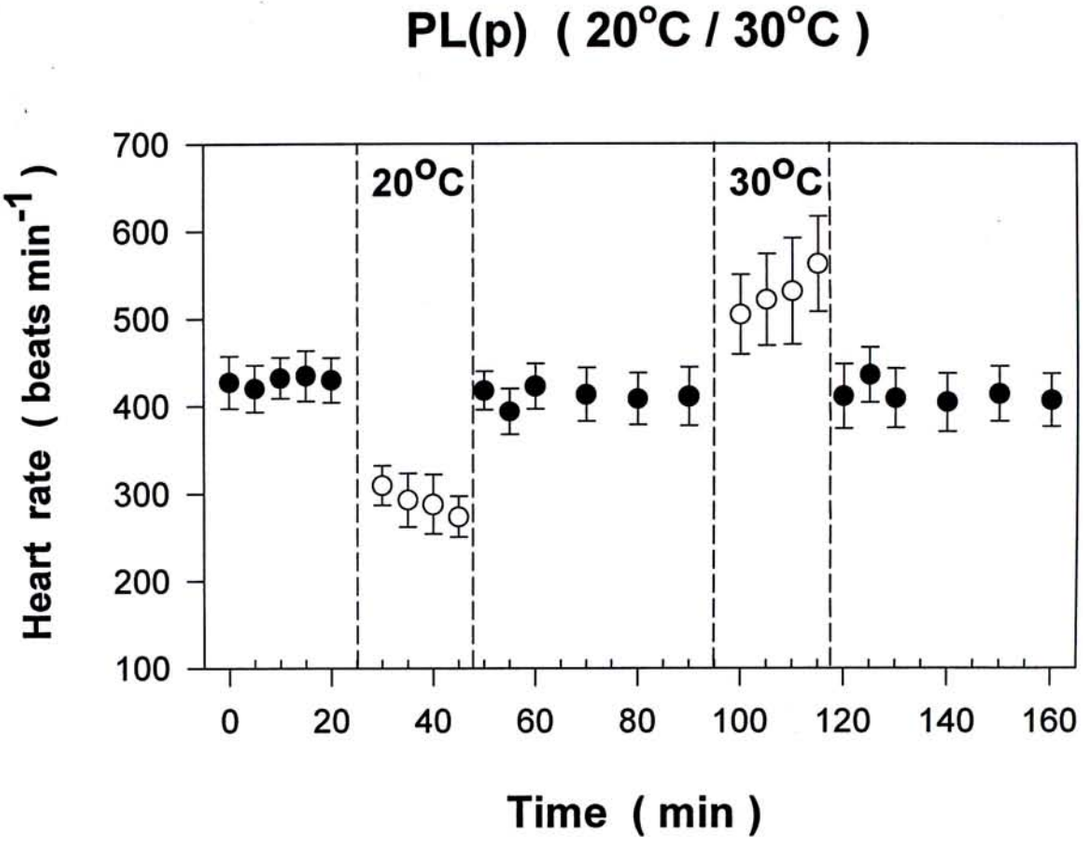


Fig. 5.5 [a] Change in heart rate of PL(p) (planktonic postlarvae) reared at 25°C in response to acute exposure to 20°C first and then 30°C. [b] Change in heart rate of PL(p) (planktonic postlarvae) reared at 25°C in response to acute exposure to 30°C first and then 20°C. Values are means ± standard error, n = 5. Dotted lines indicate when the temperature changed. Values within each temperature period are not significantly different from each other ($p>0.05$). Values of closed circles are not significantly different from the values at the acclimation period ($p>0.05$) and values of open circles are significantly different from the values at the acclimation period ($p<0.05$; One-way ANOVA with repeated measures, followed by Student-Newmans-Keuls test).


PL(p) ( 20°C / 30°C )
Heart rate ( beats min-1 )
700
600
500
400
300
200
100
20°C
30°C
0
20
40
60
80
100
120
140
160
Time ( min )

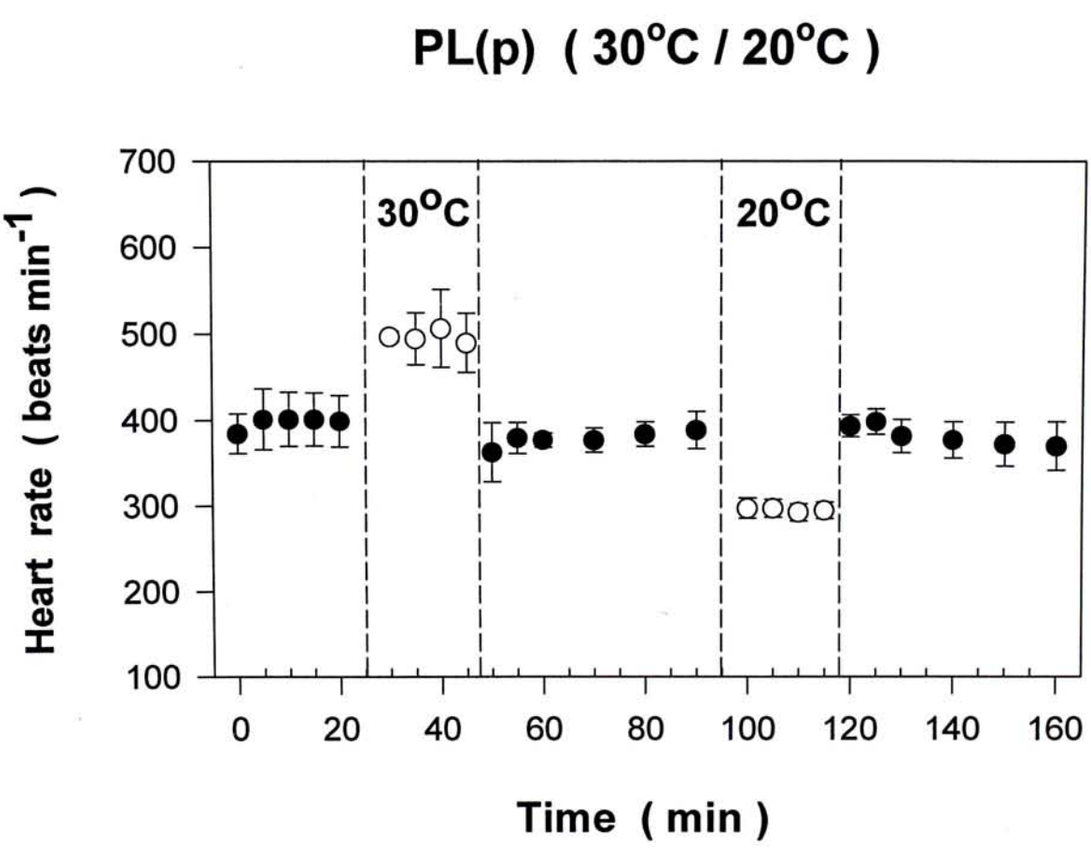
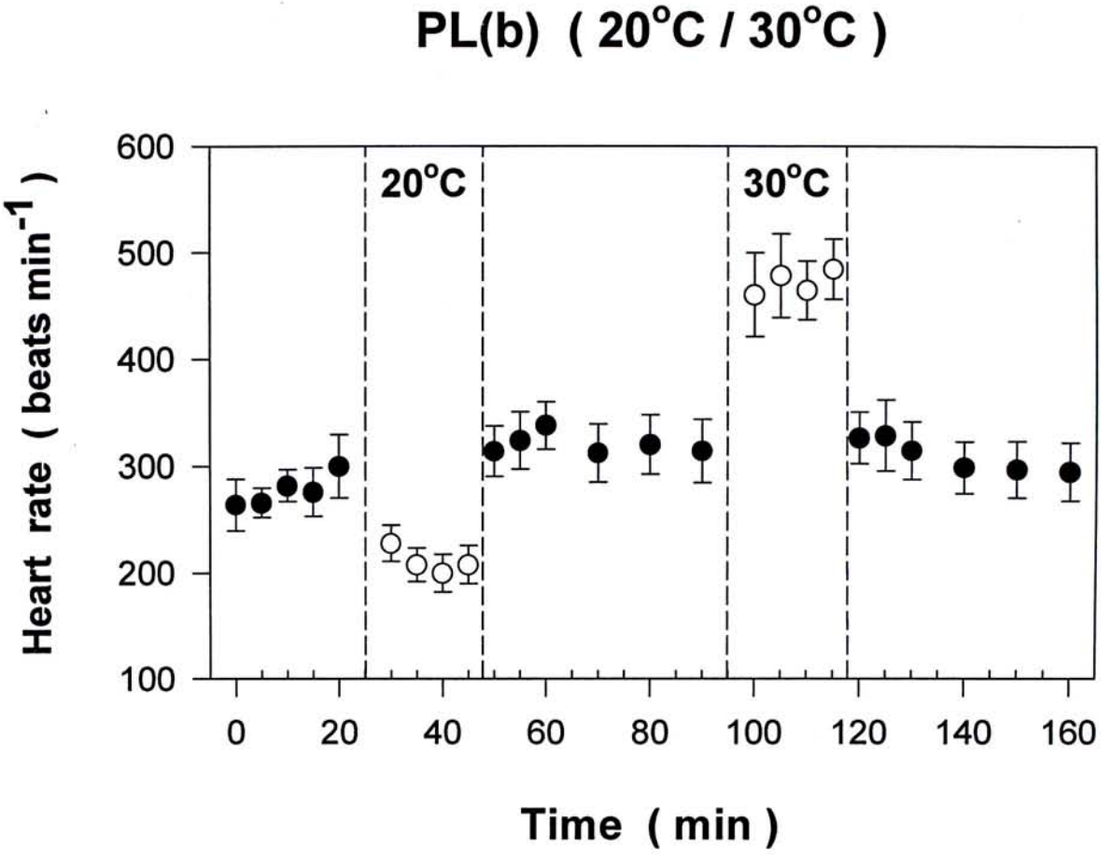

PL(p) ( 30°C / 20°C )
Heart rate ( beats min-1 )
700
600
500
400
300
200
100
30°C
20°C
0
20
40
60
80
100
120
140
160
Time ( min )

Fig. 5.6 [a] Change in heart rate of PL(b) (benthic postlarvae) reared at 25°C in response to acute exposure to 20°C first and then 30°C. [b] Change in heart rate of PL(b) (benthic postlarvae) reared at 25°C in response to acute exposure to 30°C first and then 20°C. Values are means ± standard error, n = 5. Dotted lines indicate when the temperature changed. Values within each temperature period are not significantly different from each other ($p>0.05$). Values of closed circles are not significantly different from the values at the acclimation period ($p>0.05$) and values of open circles are significantly different from the values at the acclimation period ($p<0.05$; One-way ANOVA with repeated measures, followed by Student-Newmans-Keuls test).


PL(b) ( 20°C / 30°C )
Heart rate ( beats min-1 )
600
500
400
300
200
100
20°C
30°C
0
20
40
60
80
100
120
140
160
Time ( min )

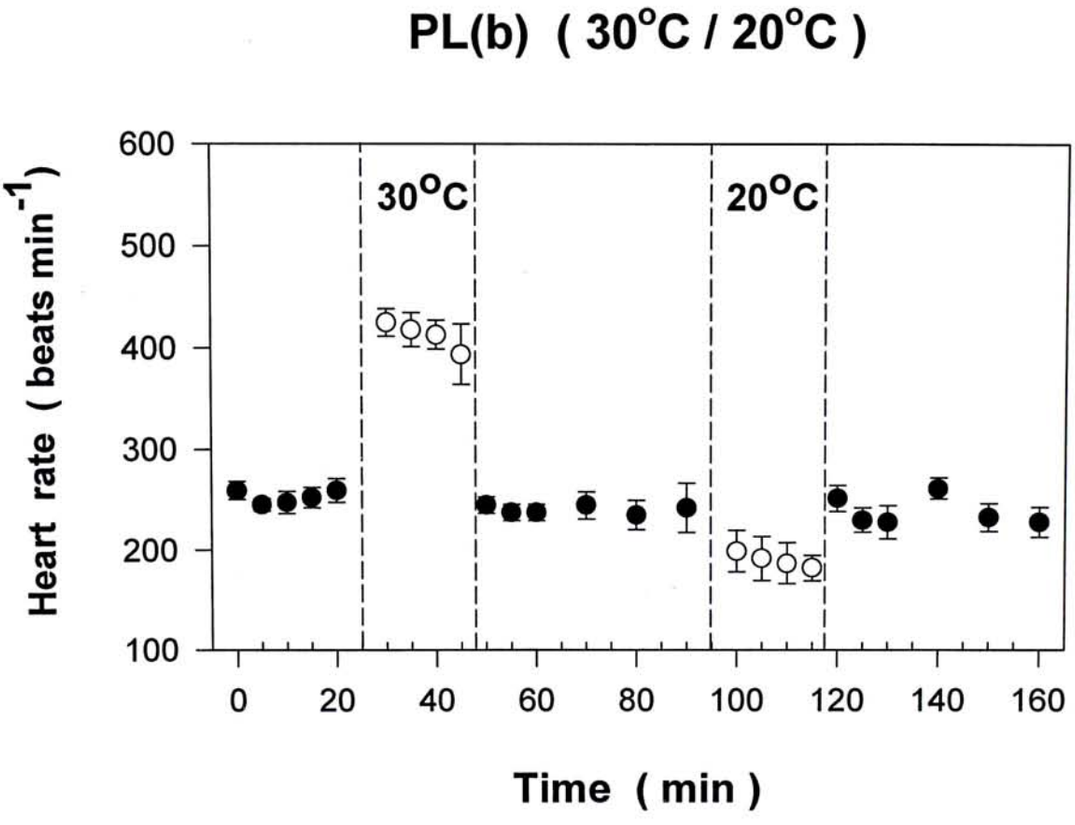

PL(b) ( 30°C / 20°C )
Heart rate ( beats min-1 )
600
500
400
300
200
100
30°C
20°C
0
20
40
60
80
100
120
140
160
Time ( min )

Table 5.1 $Q_{10}$ between 20 to 25°C and between 25 to 30°C from experiment with cold exposure first or hot exposure first. Values are means ± standard error, n = 5. The asterisks denote the value of $Q_{10}$ determined in experiment with 30°C exposure first is significantly different from the value determined in experiment with 20°C exposure first ($p < 0.05$; Student's t-test).

| Developmental Stage | $Q_{10}$ between 20 to 25°C | | $Q_{10}$ between 25 to 30°C | |
|---|---|---|---|---|
| | 20°C exposure first | 30°C exposure first | 20°C exposure first | 30°C exposure first |
| PZIII | 1.37 ± 0.04 | 2.18 ± 0.16* | 0.62 ± 0.07 | 0.61 ± 0.13 |
| MIII | 2.07 ± 0.20 | 2.36 ± 0.10 | 1.17 ± 0.19 | 0.85 ± 0.13 |
| PL(p) | 2.33 ± 0.33 | 1.67 ± 0.12 | 1.75 ± 0.25 | 1.83 ± 0.55 |
| PL(b) | 1.60 ± 0.09 | 1.90 ± 0.36 | 2.15 ± 0.15 | 2.69 ± 0.21 |

Table 5.2 $Q_{10}$ between 20 to 25°C and between 25 to 30°C of selected ontogenetic stages. Values are means ± standard error. The numbers inside the brackets indicate the sample size. The asterisks denote the values of $Q_{10}$ between 25 to 30°C are significantly different from the value of $Q_{10}$ between 20 to 25°C ($p < 0.05$; Student's t-test).

| Developmental Stage | $Q_{10}$ between 20 to 25°C | $Q_{10}$ between 25 to 30°C |
|---|---|---|
| PZIII | 1.37 ± 0.04 (5) | 0.06 ± 0.22* (5) |
| | 2.18 ± 0.16 (5) | |
| MIII | 2.21 ± 0.12 (10) | 1.01 ± 0.12* (10) |
| PL(p) | 2.00 ± 0.20 (10) | 1.79 ± 0.28 (10) |
| PL(b) | 1.75 ± 0.18 (10) | 2.42 ± 0.15* (10) |

ANOVA, Student-Newman-Keuls test, $P<0.05$) (Fig. 5.7; see also Table 5.3).

## 5.5 Discussion

Heart rate in crustaceans has generally been shown to increase with temperature when measured in the zone of capacity adaptation (Maynard, 1960a). This is related to metabolic rate of poikilotherms which is proportional to temperature. As oxygen consumption increases with temperature but oxygen affinity of hemocyanin decreases with increase in temperature (Zainal, 1992), oxygen demand may be met by an increase in heart rate. In fact, numerous studies showed that the heart rate of crustaceans increased with temperature over the capacity range, but beyond this no further increase was observed and the heart rate decreased or became irregular at higher temperature. Examples include the crabs *Carcinus maenas* (Ansanullah and Newell, 1971), *Cancer magister* (Florey and Kiebel, 1974; De Wachter and Wilkens, 1996), *Cancer productus* (Florey and Kiebel, 1974), the Australian crayfish *Cherax tenuimanus* (Villarreal, 1990) and the squat lobsters *Munida rugosa* and *Munida sarsi* (Zainal *et al.*, 1992). For instance, when squat lobsters *Munida rugosa* and *Munida sarsi* originally kept at 10°C were exposed to temperatures between 5 and 15°C, heart rate increased with temperature (Zainal *et al.*, 1992). Heart rate was progressively increased up to 20°C but at higher temperature (20-25°C) which is out of the capacity range of the cold-water lobsters, both animals showed progressive decrease in heart rate.

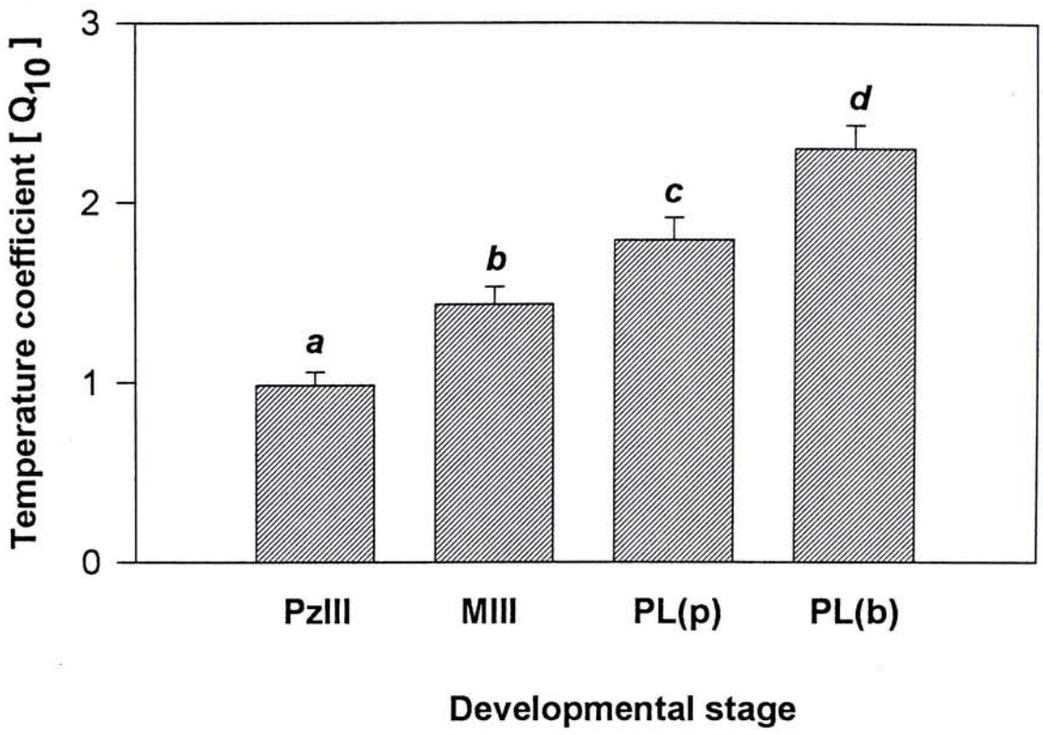



Fig. 5.7 Temperature coefficient ($Q_{10}$) between 20 to 30°C of selected ontogenetic stages. PZIII: protozoea III; MIII: mysis III; PL(p): planktonic postlarvae; PL(b): benthic postlarvae. Values are means ± standard error, n = 10. Values with different letters are significantly different ($p < 0.05$; One-way ANOVA, followed by Student-Newmans-Keuls test).

Table 5.3 $Q_{10}$ between 20 to 30°C of selected ontogenetic stages. Values are means ± standard error, n = 10. Values with different superscript letters in the same column are significantly different ($p < 0.05$; One-way ANOVA, followed by Student-Newmans-Keuls test).

| Developmental Stage | $Q_{10}$ between 20 to 30°C |
|---|---|
| PZIII | $0.98 \pm 0.07^{a}$ |
| MIII | $1.43 \pm 0.10^{b}$ |
| PL(p) | $1.79 \pm 0.12^{c}$ |
| PL(b) | $2.29 \pm 0.13^{d}$ |

The results in the present study showed that the heart rate of all four developmental stages of shrimp studied decreased in response to a fall in temperature to 20°C but exhibited variable responses to temperature increase to 30°C. The heart rate of protozoea III decreased in response to 30°C and the heart rate of mysis III either decreased slightly or did not respond to 30°C. On the other hand, the heart rate of postlarvae increased in response to 30°C. The responses of postlarvae to temperature change are similar to those of the adult crustaceans within their temperature capacity range, while the responses of larvae are similar to those of the adult crustaceans outside their temperature capacity range. The results suggested that the temperature capacity range may vary with developmental stage. This may be correlated to the life cycle of penaeid shrimp. Protozoea III and mysis III stages inhabit offshore waters where temperature is lower and less variable but postlarvae start to migrate to inshore water where temperature fluctuates to a greater extent and they have to be more tolerant. A number of studies showed that optimal temperature conditions for the survival and growth of estuarine crustacean larvae may change markedly during development (Broekhuysen, 1937; Sandoz and Rogers, 1944; Costlow *et al.*, 1960, 1962, 1966; Lance, 1964). Early developmental stages of *Metapenaeus ensis* may have the similar change in optimal temperature in which the optimum temperature increases as the shrimp develops. The larvae may have a narrow temperature capacity range and a lower optimum temperature. A temperature of 30°C may exceed their capacity range and consequently the heart rate decreased or did not respond. In contrast, postlarvae may have a wider temperature capacity range and a higher optimum temperature. A temperature of 30°C was within the capacity range thus the heart rate increased with temperature.

The different responses between larvae and postlarvae to acute exposure to 30°C may also be related to the ontogenic changes in cardiac function. Ontogenic changes in cardiac function are associated with organogenesis as well as with growth. As the shrimp develops, the heart increases in size, changes in shape (see Fig. 3.3) and may become more complexly innervated. More extensive extrinsic control including neural and neurohormonal control may then be imposed on the heart, allowing the postlarvae to be better regulated. De Wachter and Wilkens (1996) showed that heart rate in intact *Cancer magister* increased with temperature up to 20°C. The rise in heart rate of semi-isolated heart was only one-third that in intact heart. The heart rate of semi-isolated heart increased with temperature to about 18°C and then became irregular. The differences in cardiac performance between intact heart and semi-isolated heart reflects extrinsic control in intact animals, including modulation by cardioregulatory nerves or neurohormonal modulation of the cardiac ganglion, myocardial contractility or changes in outflow resistance. Responses of protozoea III and mysis III to temperature increase may be similar to that in semi-isolated heart while response of postlarvae is similar to that in intact heart. Previous studies have shown that arthropod hearts are myogenic early in development and later become neurogenic (Yamagishi, 1990; Yamagishi and Hirose, 1992). Examples include the horseshoe crab *Limulus* (Carlson and Meek, 1908), the isopod *Ligia exotica* (Yamagishi, 1990) and the crayfish *Procambarus clarkii* (Wojciechowski and McMahon, in preparation). In *Metapenaeus ensis*, the suspected transitional stage is mysis II and III (see Chapter 3 for details). Thus the protozoea III and mysis III stages tested possibly had no neural control while

postlarvae had intrinsic control by cardiac ganglion as well as extrinsic control including cardioregulatory nerves and neurohormones, allowing the heart to be better regulated in response to increase in temperature. Investigation of heart innervation throughout development may indicate a link between cardiac innervation and ontogenic changes in cardiac function.

De Wachter and Wilkens (1996) suggested that the possible mechanisms for the limitation in heart rate increase and the decrease in heart rate at extreme temperature may be related to the fact that at these high temperatures, the muscles of the myocardium and of the ostial valves fail to contract effectively and perfusate exit via the ostia. Another explanation is that although the rhythm generator still works at these high temperatures, the motorneuronal bursts are more and more poorly translated into contractions of the cardiac muscles. As the present study shows, this is not related to structural damage to enzyme systems because a decrease in temperature restores the heart to its original functionality. The possible mechanisms stated above may also apply to early developmental stages of *Metapenaeus ensis*.

The $Q_{10}$ values between 20 and 30°C increased from 0.98±0.07 in protozoea III to 2.29±0.13 in benthic postlarvae. The magnitude of increase in metabolic rate in response to temperature changes increased as the shrimp develops. This implies that the shrimp have a better system of regulation on the heart as it develops. The results further support the explanation described above concerning life cycle of *Metapenaeus ensis*, ontogenic changes in temperature capacity range and optimum temperature. Many studies have used $Q_{10}$ to reflect the effect of temperature on

reaction rates. Heart rate in a variety of crustaceans increases with temperature to yield $Q_{10}$ values between 4 at low temperatures and 1.5 at high temperatures (Ahsanullah and Newell, 1971. Florey and Kriebel, 1974; Spaargaren, 1974; Spaargaren and Achituv, 1977; McMahon, *et al.*, 1978; deFur and Mangum, 1979; Burton *et al.*, 1980; Depledge, 1984; McMahon and Burnett, 1990; Zainal *et al.*, 1992). The temperature coefficient $Q_{10}$ values of PZIII and MIII between 20 and 25°C and those between 25 and 30°C are similar to values for adults reported in the previous studies in which higher $Q_{10}$ values were found at low temperature and lower $Q_{10}$ values were found at high temperature. The values indicate that reaction rates have a greater response to temperature change at low temperature. Additionally, a reduction in $Q_{10}$ approaching the upper temperature limit has also been interpreted as indicating that the rate of increase of metabolic rate with increase in temperature cannot be sustained (Varo *et al.*, 1991). The $Q_{10}$ value of protozoea III between 25 and 30°C is 0.06±0.02 which indicates that metabolic rate decreased with increasing temperature, while the $Q_{10}$ value of mysis III between 25 and 30°C is 1.01±0.12 which indicates that metabolic rate did not change with increasing temperature. The $Q_{10}$ in protozoea III and mysis III showed that there is a decrease in temperature sensitivity with increasing temperature in both stages. However, the $Q_{10}$ value between 20 and 25°C of planktonic postlarvae was not significantly different from the corresponding values measured between 25 and 30°C while the $Q_{10}$ value of benthic postlarvae between 20 and 25°C was lower than the corresponding values measured between 25 and 30°C. The values imply that there is no change in temperature sensitivity with temperature in planktonic postlarvae between 20 and 30°C but an increase in temperature sensitivity was found in benthic postlarvae. A

study on temperature effect on semi-isolated heart and intact heart of the crab *Cancer magister* showed that semi-isolated heart and intact heart had different $Q_{10}$ values (by De Wachter and Wilkens, 1996). The different $Q_{10}$ values suggested that the semi-isolated heart which only had intrinsic control had a lower temperature sensitivity while the intact heart which had both intrinsic and extrinsic control had a higher temperature sensitivity. As discussed before, postlarvae possibly have intrinsic as well as extrinsic control which allow them to respond more readily to temperature change and perform better heart rate regulation than the larvae which possibly had no neural control.

The results from the present study showed that the heart rate response of *Metapenaeus ensis* larvae and postlarvae to acute decrease in ambient temperature (20°C) is the same. However, heart rate response of larvae to acute increase in ambient temperature (30°C) resembles that of the crustaceans measured outside the temperature capacity range while the heart rate responses of postlarvae to acute increase in ambient temperature resembles that of the crustaceans measured within the temperature capacity range. The different responses in different early developmental stages are possibly related to ontogenic changes in temperature capacity range, optimum temperature and control of cardiac function. Larvae of *Metapenaeus ensis* inhabit offshore waters where temperature was lower and less variable, they probably had a narrow temperature capacity range and a lower optimum temperature. In contrast, postlarvae start to migrate to inshore waters where temperature fluctuates to a greater extent, they possibly have a wider temperature capacity range and a higher optimum temperature. Moreover, the

suspected transition stage for the heart change from myogenic to neurogenic is mysis II and III, the postlarvae may become more complexly innervated and receives extrinsic control including neural and neurohormonal control for better heart rate regulations.

# CHAPTER 6

# DEVELOPMENT OF HEART INNERVATION IN *METAPENAEUS ENSIS*

## 6.1 Introduction

The heart of adult crustaceans is neurogenic with a cardiac ganglion intrinsic to the heart serving as the autogenic pacemaker (McMahon and Wilkens, 1983). The nerve cells in the cardiac ganglion are controlled by the central nervous system to initiate heart pulsation. In myogenic heart, heart pulsation is not initiated by nerve impulses but controlled by pacemarker activity within the heart itself (Florey, 1966). Pacemarkers are modified muscle cells which undergo periodic self-excitation and pass on this excitation to the other muscle cells of the heart thus causing heart pulsation. There have been studies suggesting that crustacean hearts are myogenic very early in development and later become neurogenic. Examples are the lobster *Homarus americanus* (Herrick, 1909), the isopod *Ligia exotica* (Yamagishi, 1990) and the crayfish *Procambarus clarkii* (Wojciechowski and McMahon, in preparation).

In order to explore the possibility of a link between cardiac innervation and changes in cardiac function during embryonic development, there is a need of investigation on heart innervation in developing crustaceans. This chapter presents

results from a preliminary study on the heart innervation during development in *Metapenaeus ensis* using diI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; diI$C_{18}$(3) from Molecular Probe, Eugene) labeling and Mayer's haematoxyline and eosin (H & E) staining. The fluorescent dye diI is a lipophilic dye which was reported to be able to incorporate into plasma membrane of nerve cells and diffuse to the tips of axons (Honig and Hume, 1986; Godement *et al.*, 1987). Therefore, it can be used to trace the neural pathway from cell body to the axon terminals, or vice versa. The dye has been used to label neurons in mouse and chicken embryos (Godement *et al.*, 1987). Yet no attempts have been made to use diI as a neural tracer in crustaceans. The objectives of the present study are to demonstrate the use of diI as a neural tracer in the shrimp *Metapenaeus ensis* and to examine the heart innervation during development of the shrimp.

## 6.2 Material and Methods

To study the innervation of the shrimp during development, diI labeling was done in protozoea III (PZIII), mysis III (MIII), planktonic postlarvae [PL day 2 to 4: PL(p)], benthic postlarvae [PL day 7 to 9: PL(b)] and juvenile shrimp with body weight of about 0.02 g. The shrimp of selected developmental stages were fixed by immersing into freshly prepared 4% buffered paraformaldehyde at pH 7.4. The fixed shrimp were stored in 4°C for at least 2 days. In a series of experiment, several holes were made along the ventral midline of the thorax, through which a diI granule was inserted into the ventral midline which was the origin of nervous system in the

shrimp. After the dye labeling, the shrimp were transferred to 2% buffered formalin and then stored in dark at room temperature for 4 to 7 days depending on the size of the shrimp. After that, PZIII, MIII, PL(p) and PL(b) were mounted on glass slide with 0.1M phosphate buffer at pH 7.4, and then examined under the confocal microscope (MRC 600, Bio-Rad). The digital images were photographed on black and white film (Ilford 100) using a high resolution monitor. Because of the large size of the juvenile shrimp, sectioning was needed for clear examination. Shrimp were embedded in gloop (gelatin albumen mixture) and 25% glutaraldehyde (Grade II, Sigma) (10:1, v/v). Cross sections of juvenile shrimp were cut at a thickness of 100 μm using a vibratome. The cross sections were then mounted on glass slide and examined under confocal microscope as described for PZIII, MIII, PL(p) and PL(b). In order to find out the respective innervation of each of the 5 thoracic ganglia and supraesophageal ganglion, juvenile shrimp were used for another series of experiments. DiI granules were inserted precisely into thoracic ganglion 1 to 5 and the supraesophageal ganglion of the shrimp each at a time following the procedures described above, and the procedures after labeling were the same as that described above .

To study the innervation of the shrimp, Mayer's haematoxyline and eosin (H & E) staining was also done in juvenile shrimp. The shrimp were fixed by immersing into freshly prepared 4% buffered paraformaldehyde at pH 7.4. The fixed shrimp were stored in 4°C for 2 days. Dehydration was carried out in an ascending series of ethanol (70%, 80%, 95%, 2 changes for absolute ethanol, 30 min for each change). Shrimp were then cleared in xylene and embedded in Paraplast paraffin

wax (melting point 56-68°C). The specimens were sectioned with American Optical 820 microtome into sections of 6 μm thickness. The sections were stained with Mayer's haematoxyline and eosin (H & E) and then examined under light microscope.

## 6.3 Results

Fig. 6.1 shows the section of junvenile shrimp with H & E staining which indicate the position of the heart and hepatopancreas in the cephalothorax region. The ventral nerve cord of the juvenile shrimp in which dil granules were inserted is shown in Fig. 6.2. The ventral midline from thoracic part to anterior end of the juvenile shrimp was clearly observed. Fig. 6.3 shows the details of ganglion cells found at the ventral nerve cord. They were of rounded cell bodies and centrally placed nuclei. The size of these neurons was very variable. Most of the cell bodies were about 10 μm in diameter, but some might be as large as 50 μm in diameter. Due to the small size of PZIII and MIII, dil granules could not be inserted precisely at the ventral midline, so that no discrete axons originating from the central nervous system could be reported. But the dorsal view of ventral midline could be clearly shown (Fig. 6.4). In PL(p), PL(b) and juvenile shrimp, a pair of nerves was found going towards the position of the heart (Fig. 6.5). Results from H & E staining are shown in Fig. 6.6. Similarly, a pair of nerves was found going towards the position of the heart. The results further support the findings from dil labeling. The muscle around the heart also lighted up (Fig. 6.7). The muscle probably was in contact

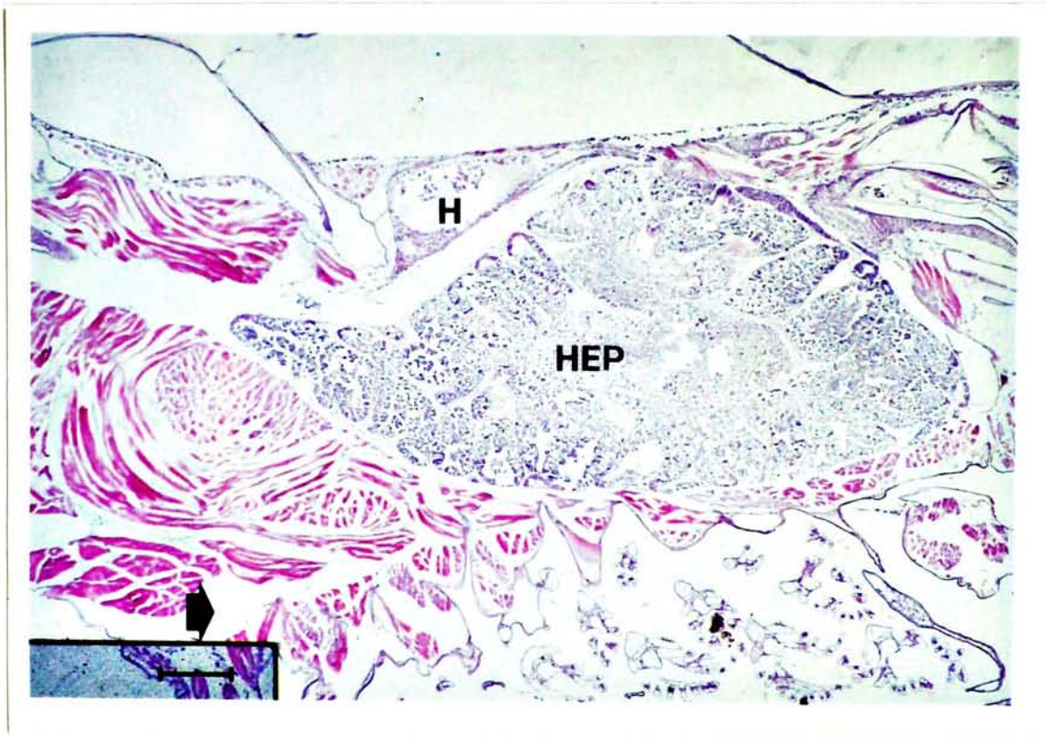



Fig. 6.1 Section showing the heart (H) and hepatopancreas (HEP) of juvenile shrimp. Arrow on the scale bar indicates the anterior direction. Longitudinal section. H& E staining. Scale bar represents 100μm.

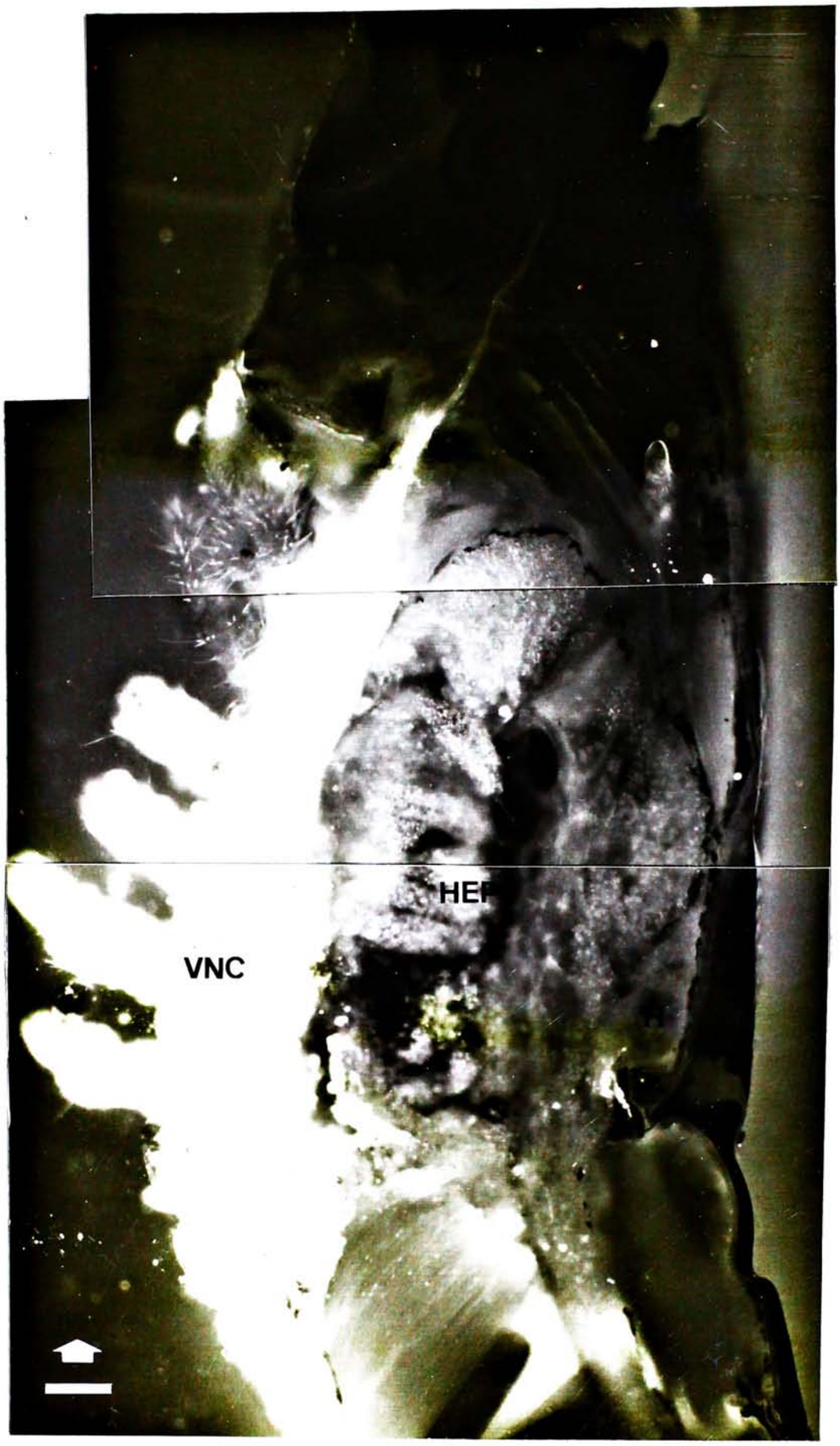



Fig. 6.2 Section showing the heart (H), hepatopancreas (HEP) and ventral nerve cord (VCN) of the juvenile shrimp. Arrow on the scale bar indicates the anterior direction. Longitudinal section. DiI labeling. Scale bar represents 100μm.

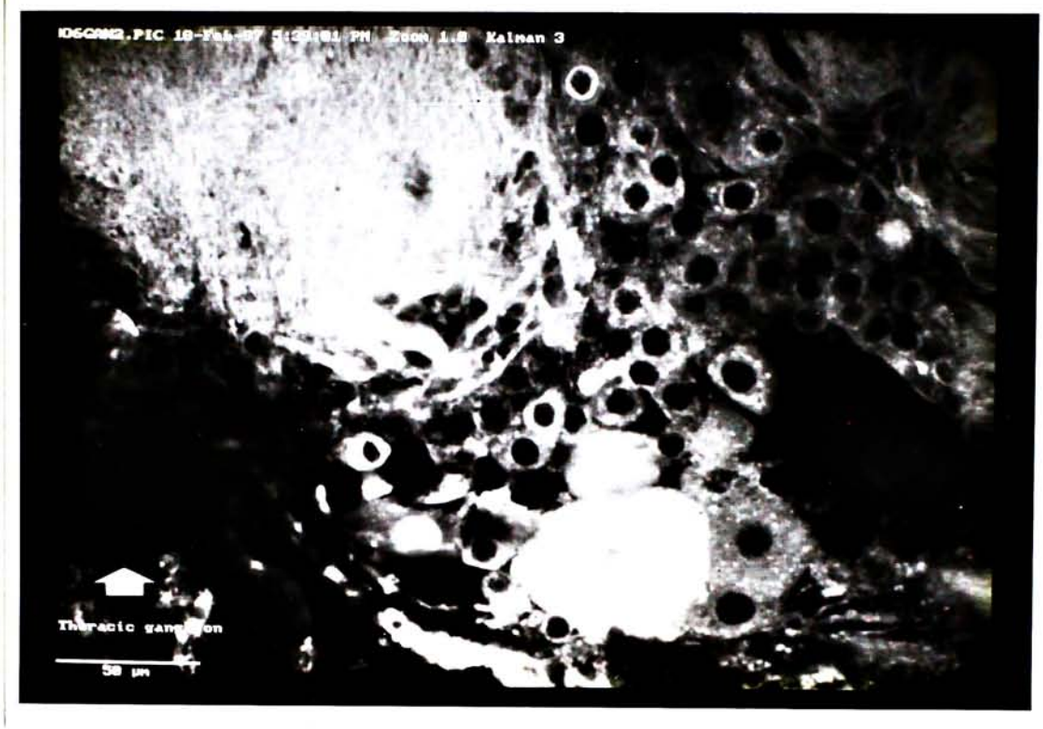



Fig. 6.3 Confocal image showing the thoracic ganglion cells found at the ventral nerve cord of benthic postlarvae. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 50 µm.

Fig. 6.4a. Confocal image showing the dorsal view of the anterior part of ventral nerve cord in protozoea III. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 100 μm.

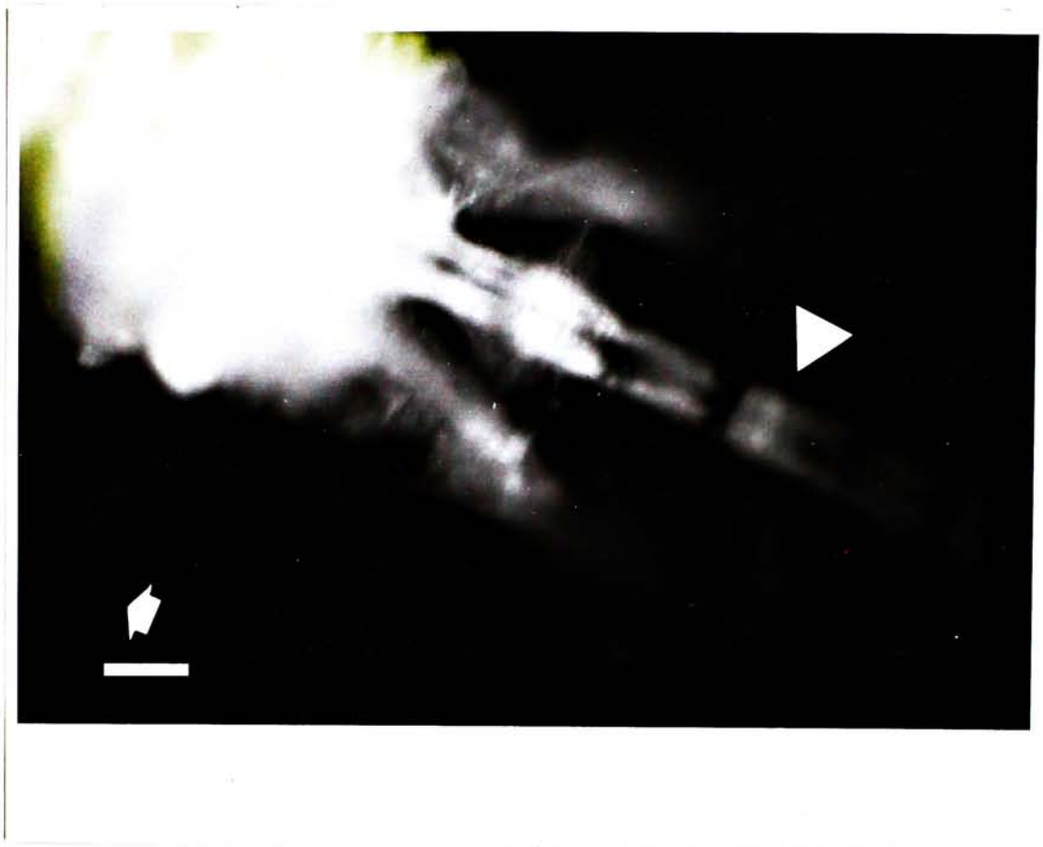


Fig. 6.4b. Section showing dorsal view of the posterior part of ventral nerve cord in protozoea III. Arrow on the scale bar indicates the anterior direction. Longitudinal section. DiI labeling. Scale bar represents 100 μm.


Fig. 6.5a. Confocal image showing a pair of nerve bundle originated from thoracic ganglia 3 and 4 go towards the position of the heart in benthic postlarvae. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 250 µm.

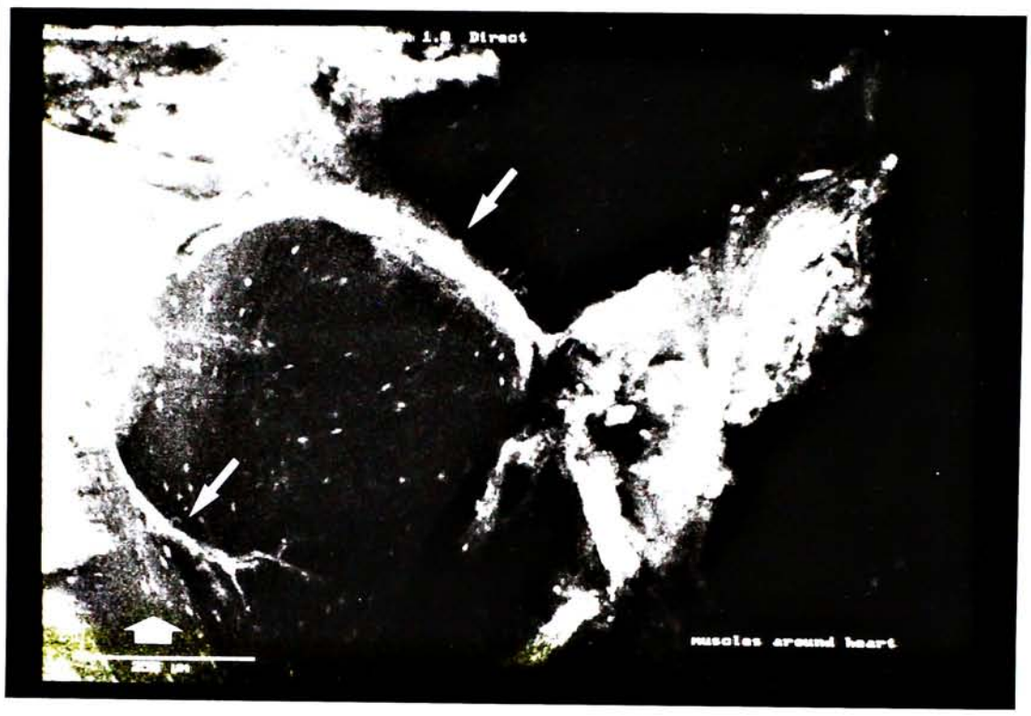



Fig. 6.5b Confocal image showing a pair of nerve bundle originated from thoracic ganglion 3 and 4 go towards the position of the heart in benthic postlarvae. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 250 μm.

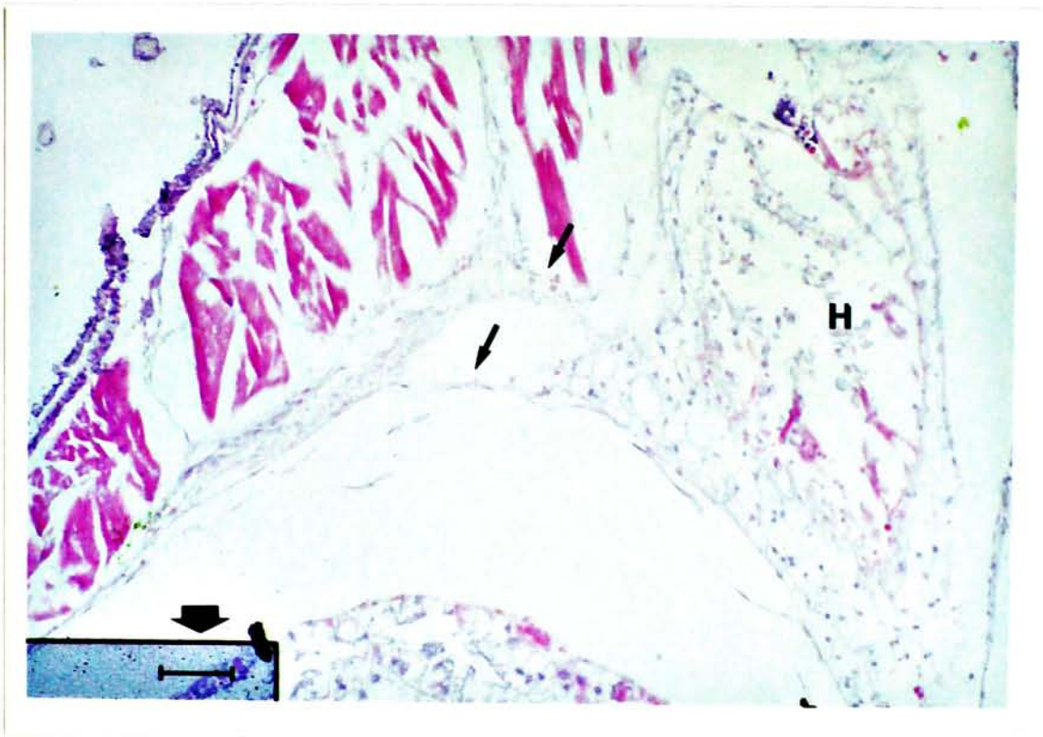



Fig. 6.6 Section showing a pair of nerve bundle originated from thoracic ganglion 3 and 4 go towards the position of the heart (H) in juvenile shrimp. Arrow on the scale bar indicates the anterior direction. Longitudinal section. H&E staining. Scale bar represents 100 μm.

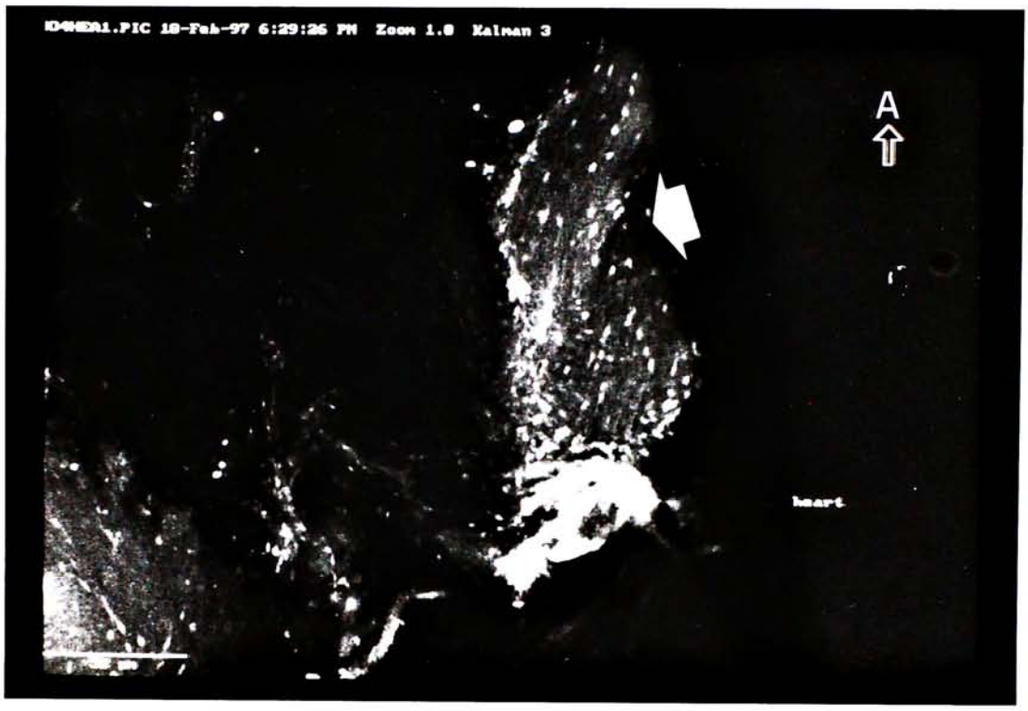



Fig. 6.7 Confocal image of the muscle around the heart and the muscle fibers at the posterior end of the heart in benthic postlarvae. Arrow with the letter A bar indicates the anterior direction. DiI labeling. Scale bar represents 50 μm.

with the dil labeled nerve bundles. Besides, the fibers at the posterior end of the heart were also labeled with dil. The structure of these fibers was quite different from ordinary muscle fibers in which the striation pattern was different (Fig. 6.8). Fig. 6.9 shows that the esophageal ring around the esophagus was also lighted up though no direct pathway was seen originated from ventral nerve cord. Fig. 6.10a shows a pair of abdominal nerves originated from the abdominal ganglion.

In the series of experiments in which dil granules were inserted precisely into thoracic ganglion 1 to 5 and the supraesophageal ganglion of juvenile shrimp, there was no conspicuous fiber from thoracic ganglion 1 and 2 that went towards the heart but there was a nerve bundle that originated from ganglion 2 to the hepatopancreas (Fig. 6.11). There were also numerous ganglion cells around the hepatopancreas (Fig. 6.12). For those shrimp which had dil inserted at thoracic ganglion 3 or 4, a pair of nerves that went close to the position of the heart was found (Fig. 6.13). The fibers from thoracic ganglion 5 mainly went to the abdominal region (Fig. 6.14). There were several nerve fibers coming out from supraesophageal ganglion. These fibers had no direct pathway to the heart and they stopped somewhere at the anterior part of the thorax (Fig. 6.15). Yet there was one nerve bundle that went very close to the hepatopancreas (Fig. 6.16).


Fig. 6.8 Confocal image showing the detailed structure of the muscle fibers at the posterior end of the heart in benthic postlarvae. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 50 μm.

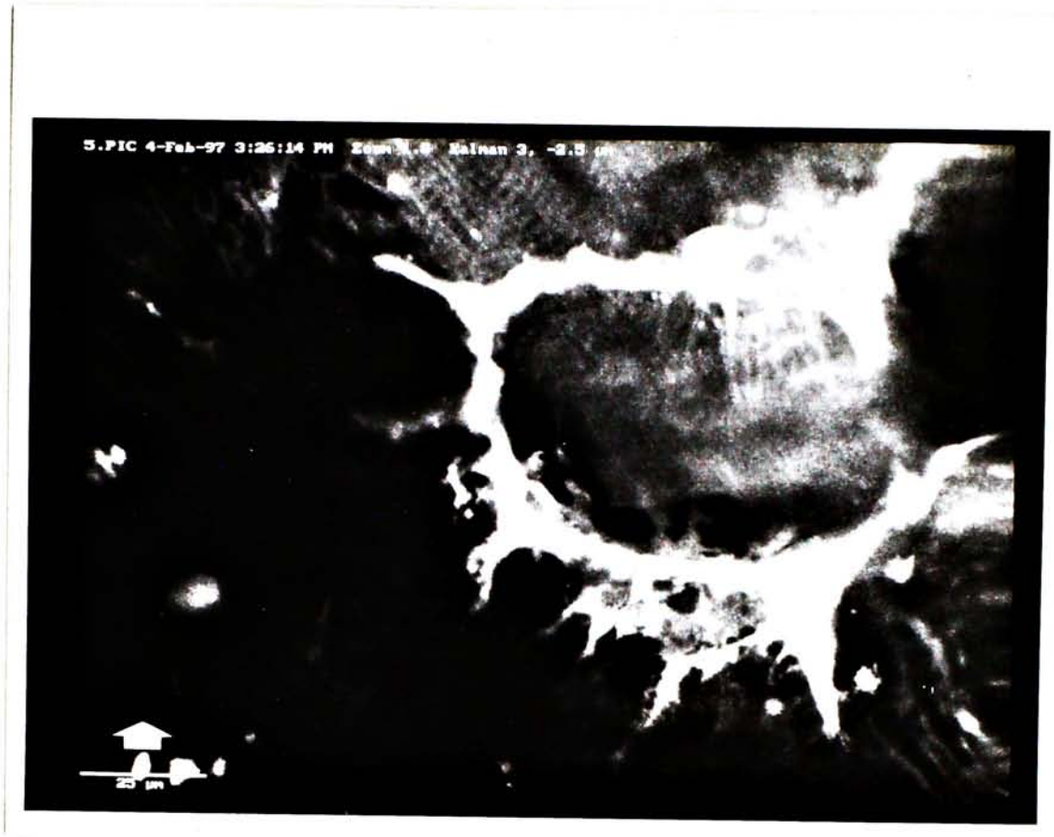



Fig. 6.9 Confocal image showing the esophageal ring in planktonic postlarvae. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 25 μm.


Fig. 6.10 Confocal image showing a pair of abdominal nerves originated from the abdominal ganglion of juvenile shrimp. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 100 µm.


Fig. 6.11 Confocal image showing the nerve bundle originated from ganglion 2 to hepatopancreas (HEP) in juvenile shrimp. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 250 μm.


Fig. 6.12 Confocal image showing the ganglion cells around the hepatopancreas (HEP) in benthic postlarvae. Arrow with the letter A indicates the anterior direction. DiI labeling. Scale bar represents 100 μm.

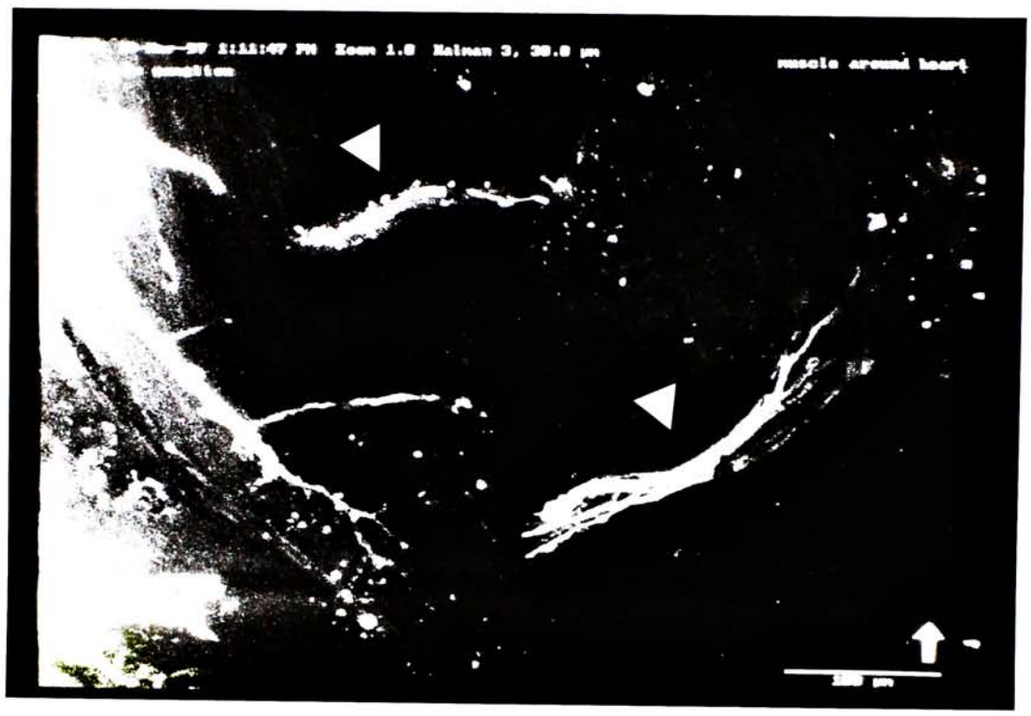



Fig. 6.13 Confocal image showing a pair of nerve bundle originated from ganglion 3 and 4 go towards to the position of the heart in juvenile shrimp. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 100 μm.

Fig. 6.14 Confocal image showing the nerve bundles originated from thoracic ganglion 5 go towards to the abdominal region in juvenile shrimp. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 500 μm.


Fig. 6.15 Confocal image showing the nerve bundles originated from supraesophageal ganglion (SG) in juvenile shrimp. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 250 μm.

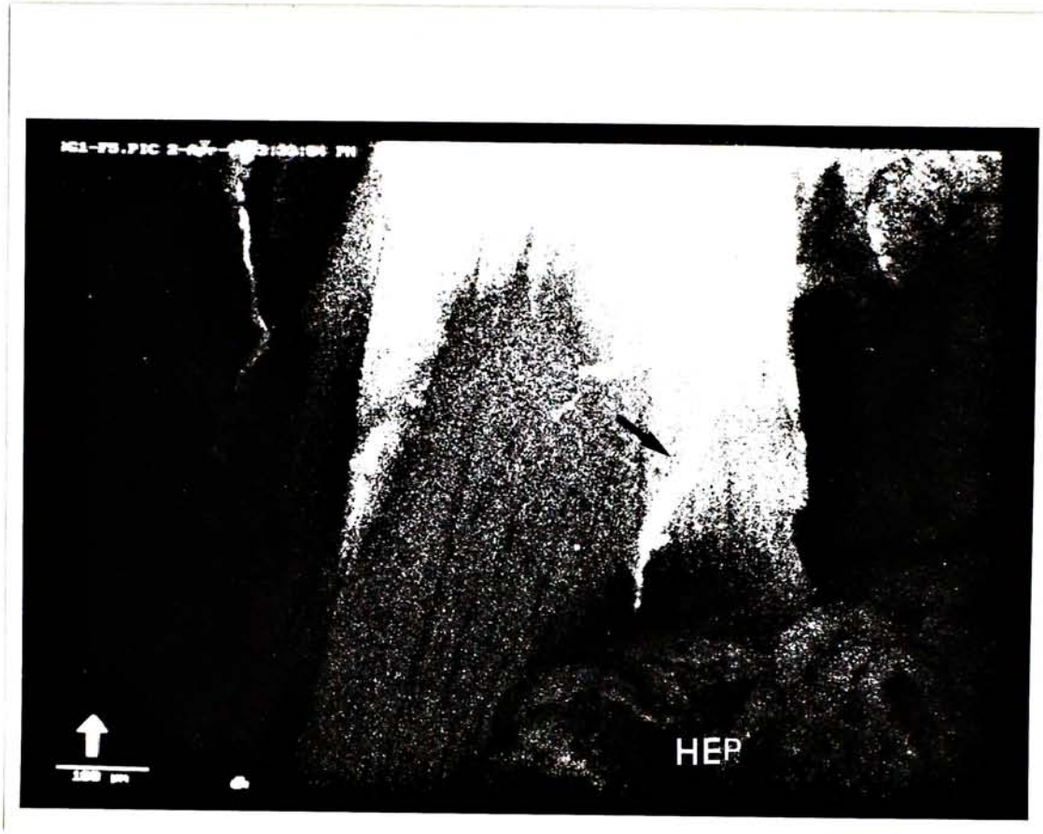



Fig. 6.16 Confocal image showing the nerve bundle originated from supraesophageal ganglion go towards to the hepatopancreas (HEP) in juvenile shrimp. Arrow on the scale bar indicates the anterior direction. DiI labeling. Scale bar represents 100 μm.

## 6.4 Discussion

The cardiac ganglion of crustaceans receives extrinsic nerve fibers via the paired dorsal nerves that arise from the central nervous system (Alexandrowicz, 1932). Each dorsal nerve contains two accelerator axons and one inhibitory axon. In isolated hearts, stimulation of the acceleratory nerves speeds the contraction rate, and stimulation of the inhibitory nerves slows or stops the heart (Maynard, 1953; Florey, 1960; Wilkens and Walker, 1992). The cardioregulatory nerves arise from the subesophageal ganglion in Macura and homologous regions of the thoracic ganglion in Brachyura (McMahon *et al.*, 1997, review). These neurons are driven, at least in part, by command interneurons descending in the circumesophageal connectives from the brain. Within the heart the regulatory neurons synapse at different locations. In isopods, the cardioinhibitory nerves synapse on both the cardiac ganglion (CG) and on the myocardium while in decapods they only innervate the CG. Since the cardioaccelerator nerves also carry axons ending in the pericardial organs, nerve stimulation proximal to the pericardial organs may produce direct synaptic excitation on the CG as well as indirect excitation via the release of neurohormones.

The present study demonstrated the use of the fluorescent dye diI as a neural tracer in the shrimp *Metapenaeus ensis*. In contrast to the results from the studies described above, no nerve fibers were found arising from supraesophageal ganglion that went towards the position of the heart. However, a pair of nerve bundles was found to arise from thoracic ganglion 3 and 4 and go towards the position of the

heart. This pair of fibers is probably involves in the control of heart function. The muscle around the heart was labeled by diI, indicating that they were in contact with the diI labeled nerve bundles. The results suggested that indirect pathways from ventral nerve cord, and the muscle may play some role in the control of heart function. The fibers at the posterior end of the heart were also labeled with diI. The structure of these fibers was quite different from ordinary muscle fibers in which the striation was much more compact. This suggested that the fibers may also take part in the control of heart function. The study on the respective innervation of each of the 5 thoracic ganglia and supraesophageal ganglion showed that each ganglion had its own innervation targets. Supraesophageal ganglion had numerous fibers going towards the posterior region of the thorax, but no discrete target region was found except the hepatopancreas. Besides supraesophageal ganglion, thoracic ganglion 2 also had fibers that innervated the hepatopancreas. Moreover, there were numerous ganglion cells around the hepatopancreas. These results suggest that hepatopancreas is highly innervated by the central nervous system. No fibers arose from thoracic ganglion 1 while the nerve fibers arising from thoracic ganglion 5 innervated the abdominal region. Thoracic ganglions 3 and 4 were the only ganglia which had nerve fibers going towards the position of the heart. This finding was further supported by the results from H & E staining. Further studies are needed to demonstrate the control of these pair of fibers on heart function.

DiI labeling provides high-resolution labeling of individual axons at discrete points. Due the limit of the diI labeling technique in the present study, the whole series of cardiac innervation during development cannot be achieved. Yet diI

labeling was confirmed to be applicable as a neural tracer in crustaceans as it does in vertebrates. To study the innervation in protozoeal and mysid stages which had a small size restriction for the insertion of the dil at the ventral midline, the technique of microinjections can be considered. Honig and Hume (1989) reported that dil can be dissolved in dimethylformamide (DMF). With fine-tipped microelectrodes and very short duration pulses of pressure, it is possible to label just a single cell in an embryo of an organism. Moreover, electrical stimulation may apply to confirm whether the pair of fibers arose from thoracic ganglions 3 and 4 take part in controlling heart function and to investigate whether they are inhibitory or excitatory. If the above information is available, it would be possible to confirm the change of myogenic heart to neurogenic heart in the shrimp *Metapenaeus ensis* as suggested in Chapter 3.

# CHAPTER 7

# CONCLUSIONS

Based on the experimental results in previous chapters, conclusions of the present study would be drawn as follows:

**1. Change in heart rate during development**

Heartbeat in *Metapenaeus ensis* was first observed in nauplius VI instar. The heart showed non-rhythmic beating until protozoea III instar. The heart rate increased dramatically from protozoea I and reached a peak at protozoea III but declined gradually from mysis I onwards. High oxygen consumption due to high swimming activity and fast developmental rate may explain why protozea III had the highest heart rate among all stages studied. A reduction in activity may contribute to a lower oxygen consumption, leading to lower heart rates observed in mysid and postlarval stages.

**2. Relationship between heart rate and body weight during development**

A biphasic relationship was found between heart rate and body weight during larval development. Heart rate increased at 1.70 power of body weight from nauplius VI to protozoea III and decreased at 0.34 power of body weight from protozoea III to benthic postlarvae. The results suggest that the decrease in heart rate

does not depend solely on the increase in body weight and other factors related to development may affect the heart rate.

**3. Change in heart shape during development**

The shape of the heart changed during larval development. The heart changed from a kite-shape in the late naupliar and protozoeal instars to a bottom flattened shape at mysid instars, and then to a rectangular shape at postlarvae. The change in the shape of the heart is possibly related to the development of blood vessels and alary ligaments which stretch the heart into a different shape. Thus the change in the shape of the heart may represent different stages of the development of the circulatory system.

**4. Change from myogenic heart to neurogenic heart**

Starting from stage mysis II and III, heartbeat of the shrimp would stop for a few milliseconds in response to stimulus. This response suggest that mysis II and III was the suspected transitional stage from a myogenic heart in earlier stages to a neurogenic heart in adult.

**5. Effect of salinity variation on heart rate during development**

The heart rate of larvae and early postlarvae reared at a salinity of 18‰ was higher than those reared at 28‰ while the heart rate of benthic postlarvae was not different at the two salinities. The different responses to low salinity appear to correlate with the increase in salinity tolerance and development of osmoregulatory ability. The larvae are probably osmoconformers while the osmoregulatory ability of

early postlarvae is not yet fully developed. However, benthic postlarvae are better equipped with osmoregulatory structures and thus are more able to compensate for low salinity than the earlier developmental stages. In all stages studied, when shrimp reared at 28‰ were exposed to 18‰, heart rate increased. The sudden drop in salinity from 28‰ to 18‰ was stressful to shrimp in all stages. The shrimp probably tried to escape and led to an increase in oxygen consumption. However, when shrimp reared at 18‰ were exposed to 28‰, heart rate did not change in all stages except mysis III where a decrease in heart rate was observed. No increase in heart rate was ever recorded. The results suggest that the elevated heart rates which develop in acclimation to low salinity conditions do not respond readily to an acute increase in salinity. The results of the acute exposure experiments in this study suggest that the elevated heart rate under low salinity stress develops very rapidly upon a change in salinity.

**6. Effect of temperature variation on heart rate during development**

The response of heart rate in *Metapenaeus ensis* larvae and postlarvae to acute decrease in ambient temperature (20°C) is the same. However, the response of the heart rate in larvae to acute increase in ambient temperature (30°C) resembles that of the crustaceans measured outside the temperature capacity range while the response of heart rate in postlarvae to acute increase in ambient temperature resembles that of the crustaceans measured within the temperature capacity range. The different responses in different early developmental stages are possibly related to life cycle of *Metapenaeus ensis*, and ontogenic changes in temperature capacity range, optimum temperature and control of cardiac function. Larve of *Metapenaeus*

*ensis* inhabit offshore waters where temperature is lower and less variable. The larvae probably have a narrow temperature capacity range and a lower optimum temperature. In contrast, postlarvae start to migrate to inshore waters where temperature fluctuates to a greater extent. They possibly have a wider temperature capacity range and a higher optimum temperature. Moreover, since the heart appears to change from myogenic to neurogenic at mysis II and III, the postlarvae may become more complexly innervated and the heart receives extrinsic control including neural and neurohormonal control for better heart rate regulations in response to temperature change.

**7. Heart innervation during development**

DiI labeling was confirmed to be applicable as a neural tracer in crustaceans as it does in vertebrates. Though no direct pathway from ventral nerve cord to the heart was found, a pair of nerve bundle was found to originate from thoracic ganglions 3 and 4, and went close to the position of the heart. This pair of nerve bundle is possibly involved in the control of heart function. Muscle around heart and the muscle fibers at the posterior end of the heart were labeled by diI, suggesting suggested that they may receive information from the ventral nerve cord indirectly and thus may play certain role in the control of heart function.

# REFERENCES


Aagaard, A. (1996). In situ variation in heart rate of the shore crab *Carcinus maenas* in relation to environmental factors and physiological condition. *Marine Biology*, **125**:765-772.

Ahsanullah, M. and Newell, R.C. (1971). Factors affecting the heart rate of the shore crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology*, **39A**:277-287.

Airriess, C.N. and McMahon, B.R. (1992). Aminergic modulation of circulatory performance in the crab *Cancer magister*. *Comparative Physiology*, **11**:123-131.

Airriess, C.N. and McMahon, B.R. (1994). Cardiovascular adaptations enhance tolerance of environmental hypoxia in the crab *Cancer Magister*. *Journal of Experimental Biology*, **190**:23-41.

Alcaraz, M., Saiz, E. and Calbet, A. (1994). Small-scale turbulence and zooplankton metabolism: effects of turbulence on heartbeat rates of planktonic crustaceans. *Limmology and Oceanography*, **39**:1465-1470.

Alexandrowicz, J.S. (1932). The innervation of the heart of the Crustacea. I. Decapod. *Quarterly Journal of Microscopic Science*, **75**:181-249.

Anderson, M. and Cooke, I.M. (1971). Neural activation of the heart of the lobster *Homarus americanus*. *Journal of Experimental Biology*, **55**:449-468.

Audsley, N.C., Mcintosh, C. and Phillips, J.E. (1992). Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport. *Journal of Experimental Biology*, **173**:260-274.

Benson, J.A. (1981). Synaptic and regenerative responses of cardiac muscle fibres in the crab, *Portunus sanguinolentus*. *Journal of Comparative Physiology*, **143A**:349-356.

Bouaricha, N., Charmantier-Daures, M., Thuet, P., Thrilles, J.P. and Charmantier, G. (1994). Ontogeny of osmoregulatory structures in the shrimp *Penaeus japonicus* (Crustacea, Decapoda). *Biological Bulletin*, **186**:29-40.

Broekhuysen, G.J. (1937). On development, growth and distribution of *Carcinus maenas* (L.). *Archives Néerlandaises de Zoologie*, **2**:257-399.

Burton, D.T., Richardson, L.B. and Moore, C.J. (1980). Cardiac frequency compensation responses of adult blue crabs (*Callinectes sapidus* Rathbun) exposed to moderate temperature increases. *Comparative Biochemistry and Physiology*, **65A**:259-263.

Cameron, J.N. and Mangum, C.P. (1983). Environmental adaptations of the respiratory system: Ventilation, circulation, and oxygen transport. In: Vernberg, F.J., Vernberg, W.B. and Bliss, D.E. (eds.) *The Biology of Crustacea, Vol. 8. Environmental Adaptations*. New York and London: Academic Press, pp. 43-63.

Cawthrone, D.F., Beard, T., Davenport, J. and Wickins, J.F. (1983). Responses of juvenile *Penaeus monodon* Fabricius to natural and artificial sea waters of low salinity. *Aquaculture*. **32**:165-174.

Carlson, A.J. and Meek, W.J. (1908). On the mechanism of the embryonic heart rhythm in *Limulus*. *American Journal of Physiology*, **21**:1-10.

Chen, J. and Lai, S. (1993). Effects of temperature and salinity on oxygen consumption and ammonia-N excretion of juvenile *Penaeus japonicus* Bate. *Comparative Biochemistry and Physiology,* **165**:161-170.

Chu, K.H. and Ovsianico-Koulikowsky, N.N. (1994). Ontogenetic changes in metabolic activity and biochemical composition in the shrimp, *Metapenaeus ensis*. *Journal of Experimental Marine Biology and Ecology*, **183**:11-26.

Chu, K.H. and So, S.H. (1987). Changes in salinity tolerance during larval development of the shrimp *Metapenaeus ensis* (De Haan). *Asian Marine Biology*, **4**:41-48.

Chu, K.H., Mak, E.M.T. and McMahon, B.R. (1995). Effect of salinity on heart rate of larvae and postlarvae of the shrimp, *Metapenaeus ensis*. *American Zoologist,* **35**: 64A. (Abstract)

Chu, K.H., Sze, C.C. and Wong, C.K. (1996). Swimming behavior during the larval development of the shrimp *Metapenaeus ensis* (De Haan, 1844) (Decapoda, Penaeidae). *Crustaceana*, **69**:368-378.

Cornell, J.C. (1973). A reduction in water permeability in response to a dilute medium in the stenohaline crab *Libinia emarginata* (Brachyura, Majidae). *Biological Bulletin*, **145**:430-431.

Cornell, J.C. (1974). A reduction in water exchange rates in an osmoconforming crab. *American Zoologist*, **14**:1259. (Abstract)

Costlow, J.D., JR., Bookhour, C.G. and Monroe, R. (1960). The effect of salinity and cyclic temperature on larval development of *Sesarma cinereum* (Bosc.) reared in the laboratory. *Biological Bulletin*, **118**:183-202.

Costlow, J.D., JR., Bookhout, C.G. and Monroe, R. (1962). Salinity-temperature effects on the larval development of the crab *Panopeus herbstii* Milne-Edwards, reared in the laboratory. *Phsiological Zoology*, **35**:79-93.

Costlow, J.D., JR., Bookhout, C.G. and Monroe, R. (1966). Studies on the larval development of the crab *Rhithropanopeus harrisii* (Gould). 1. The effect of salinity and temperature on larval development. *Phsiological Zoology*, **39**:81-100.

Dalla Via, G.J. (1986). Salinity response of the juvenile penaeid shrimp *Penaeus japonicus* I. Oxygen consumption and estimation of productivity. *Aquaculture*, **55**:297-306.

Dalla Via, G.J. (1987). Effects of salinity and temperature on oxygen consumption in a freshwater population of *Palaemonetes antennarius* (Crustacea, Decapoda). *Comparative Biochemistry and Physiology*, **88**:299-305.

De Wachter, B. and McMahon, B.R. (1996). Temperature effects on heart performance and regional hemolymph flow in the crab *Cancer magister*. *Comparative Biochemistry and Physiology*, **114A**:27-33.

De Wachter, B. and Wilkens, J.L. (1996). Comparison of temperature effects on heart performance of the Dungeness crab, *Cancer magister*, in vitro and in vivo. *Biological Bulletin*, **190**:385-395.

DeFur, P.L. and Mangum, C.P. (1979). The effects of environmental variables on the heart rates of invertebrates. *Comparative Biochemistry and Physiology*, **62A**:283-294.

Depledge, M.H. (1984). Cardiac activity in the intertidal crab *Hemigrapsus sanguineus* (De Haan). *Asian Marine Biology*, **1**:115-123.

Dimock, R.V. and Groves, K.H. (1975). Interaction of temperature and salinity on oxygen consumption of the estuarine crab *Panopeus herbstii. Marine Biology,* **33**:301-308.

Dyer, M.F. and Uglow, R.F. (1980). Cardiac and ventilatory changes associated with altered salinity in *Crangon crangon* (L.). *Ophelia*, **19**:117-125.

Engel, D.W. and Eggert, L.D. (1974). The effect of salinity and sex on the respiration rates of excised gills of the blue crab, *Callinectes sapidus. Comparative Biochemistry and Physiology,* **47A**:1005-1011.

Farrell, A.P. (1991). Circulation of body fluid. In: Prosser, C.L. (ed.), *Environmental and Metabolic Animal Physiology.* New York: Wiley, Liss, pp. 509-558.

Findley, A.M., Belisle, B.W. and Stickle, W.B. (1978). Effects of salinity fluctuations on the respiration rate of the southern oyster drill *Thais haemastoma* and the blue crab *Callinectes sapidus*. *Marine Biology*, **49**:59-67.

Fischer, L. and Florey, E. (1981). Temperature effects on neuromuscular transmission (opener muscle of crayfish, *Astacus leptodactylus*). *Journal of Experimental Biology*, **94**:251-268.

Florey, E. (1960). Studies on the nervous regulation of the heart beat in decapod Crustacea. *Journal of General Physiology*, **43**:1061-1081.

Florey, E. and Hoyle, G. (1961). Neuromuscular synaptic activity in the crab *Cancer magister*. In: Florey, E. (ed.), *Nerve Inhibition*. Oxford, London, New York and Paris: Pergamon, pp. 105-110.

Florey, E. and Hoyle, G. (1976). The effects of temperature on a nerve-muscle system of the Hawaiian ghost crab, *Ocypode ceratophthalma* (Pallas). *Journal of Comparative Physiology*, **110**:51-64.

Florey, E. and Kriebel, M.E. (1974). The effects of temperature, anoxia and sensory stimulation on the heart rate of understrained crabs. *Comparative Biochemistry and Physiology*, **48A**:285-300.

Gaudy, R. and Sloane, L. (1981). Effect of salinity on oxygen consumption in postlarvae of the penaeid shrimps *Penaeus mondodon* and *P. stylirostris* without and with acclimation. *Marine Biology*, **65**:297-301.

Gliwicz, M.Z. and Sieniawska, A. (1986). Filtering activity of *Daphnia* in low concentrations of a pesticide. *Limnology and Oceanography*, **31**:1132-1138.

Godement, P., Vanselow, J., Thanos, S. and Bonhoeffer, F. (1987). A study in developing visual systems with a new method of staining neurons and their processes in fixed tissue. *Development*, **101**:697-713.

Green, J.W., Harsch, M., Barr, L. and Prosser, C.L. (1959). The regulation of water and salts by fiddler crabs, *Uca pugnax* and *Uca pugilator*. *Biological Bulletin*, **116**:76-87.

Hammel, H.T., Stromme, S.B. and Myrhe, K. (1969). Forebrain temperature activates behaviour thermoregulatory response in Arctic sculpins. *Science*, **165**:83-85.

Hawlett, M. (1971). Lobster heart: Electrophysiology of single cells including effects of the regulator nerves. *Comparative Biochemistry and Physiology*, **39A**:643-648.

Herrick, F.H. (1909). Natural history of the American lobster. *Bulletin. United States Bureau of Fisheries*, **29**:149-408.

Honig, M.G. and Hume, R.I. (1989). DiI and DiO: versatile fluorescent dyes for neuronal labelling and pathway tracing. *Trends in Neuroscience*, **12**:333-341.

Horridge, G.A. (1965). Arthropoda: Nervous control of effector organs. In: Bullock, T.H. and Horridge, G.A. (eds.) *Structure and Function in the Nervous Systems of Invertebrates*, Vol. 2. San Francisco: Freeman, pp.965-1004.

Hume, R.I. and Berlind, A. (1976). Heart and scaphognathite rate changes in a euryhaline crab, *Carcinus maenas*, exposed to dilute environmental medium. *Biological Bulletin,* **150**:241-254.

Jacobs, J.R. and Atwood, H.L. (1981). Long term facilitation of tension in crustacean muscle and its modulation by temperature, activity and circulating amines. *Journal of Comparative Physiology*, **144**:335-343.

Jury, S.H., Kinnidon, M.Y., Howell, W.H. and Watson, W.H. (1994a). The behavior of lobsters in response to reduced salinity. *Journal of Experimental Marine Biology and Ecology*, **180**:23-37.

Jury, S.H., Kinnison, M.T., Howell, W.H. and Waston, W.H. (1994b). The effects of reduced salinity on lobster (*Homarus americanus* Milne-Edwards) metabolism: Implications for estuarine populations. *Journal of Experimental Marine Biology and Ecology*, **176**:167-185.

Kinne, O. (1952). Zur Biologie und Physiologie von *Gammarus duebeni* Lillj. Untersuchungen Uber Blutkonzentration, Herzfrequenz ünd Atmung. *Kieler Meeresforschungen*, **9**:134-150.

Kinne, O. (1967). Physiology of estuarine organisms with special reference to salinity and temperature: general aspects. In: Kinne, O. (ed.) *Estuaries*. Washington, D.C.: American Association for the Advancement of Science. (Publs Am. Ass. Advmt Sci. No. 83), pp. 525-540.

Kinne, O. (1971). Salinity: animals-invertebrates. In: Kinne, O. (ed.) *Marine Ecology.* Vol.1. Environmental Factors, Pt. 2. London: Wiley Interscience, pp. 821-1083.

Knowlton, R.E. and Schoen, R.H. (1984). Salinity and tolerance and sodium balance in the prawn *Palaemonetes vulgaris* (Say) compared with *P. pugio*. *Comparative Biochemistry and Physiology*, **77A**: 425-430.

Krogh, A. (1938). The active absorption of ions in some fresh water animals. *Zeitschrift Vergleichende Physiologie*, **25**:335-350.

Kuramoto, T. and Ebara, A. (1984). Effects of perfusion pressure on the isolated heart of the lobster, *Panulirus japonicus*. *Journal of Experimental Biology*, **109**:121-140.

Lance, J. (1964). The salinity tolerance of some estuarine planktonic crustaceans. *Biological Bulletin*, **127**:108-118.

Leffler, Ch. W. (1975). Ionic and osmotic regulation and metabolic response to salinity of juvenile *Callinectes sapidus* Rathburn. *Comparative Biochemistry and Physiology,* **52**:545-549.

Leong, P.K.K., Chu, K.H. and Wong, C.K. (1992). Larval development of *Metapenaeus ensis* (de Haan) (Crustacea: Decapoda: Penaeidae) reared in the laboratory. *Journal of Natural History*, **26**:1283-1304.

Mantel, L. and Farmer, L.L. (1983). Osmotic and ionic regulation. In: Mantel, L.M. and Bliss, D.E. (eds.), *The Biology of Crustacea,. Vol. 5. Internal Anatomy and Physiological Regulation.* New York: Academic Press, pp. 54-61.

Maynard, D.M. (1953). Activity in a crustacean ganglion. I. Cardioinhibition and acceleration in *Panulirus argus*. *Biological Bulletin*, **104**:156-170.

Maynard, D.M. (1960a). Circulation and heart function. In: Waterman, T.H. (ed.), *The Physiology of the Crustacea. Vol. 1. Metabolism and Growth.* New York: Academic Press, pp. 161-226.

Maynard, D.M. (1960b). Heart rate and body size in the spiny lobster. *Physiological Zoology*, **13**:241-251.

Maynard, D.M. (1961). Thoracic neurosecretory structures in Brachyura. I. Gross anatomy. *Biological Bulletin*, **121**:316-329.

McGaw, I.J. and McMahon, B.R. (1996). The effects of salinity variation on regional hemolymph distribution in the Dungeness crab, *Cancer magister*. *Physiological Zoology*, **69**:1384-1401.

McGaw, I.J., Airriess, C.N. and McMahon, B.R. (1994). Patterns of hemolymph-flow variation in decapod crustaceans. *Marine Biology*, **121**:53-60.

McGaw, I.J., Wilkens, J.L., McMahon, B.R. and Airriess, C.N. (1995). Crustacean cardioexcitatory peptides may inhibit the heart in vivo. *Journal of Experimental Biology*, **198**:2547-2550.

McLaughlin, P.A. (1980). *Comparative Morphology of Recent Crustacea.* San Francisco: Freeman, pp. 1-117.

McLaughlin, P.A. (1983). Internal anatomy. In: Mantel, L.H. and Bliss, D.E. (eds.), *The Biology of Crustacea, Vol. 5. Internal Anatomy and Physiological Regulation.* New York: Academic Press, pp. 1-52.

McMahon, B.R. and Burnett, L.E. (1990). The crustacean open circulatory system: a reexamination. *Physiological Zoology*, **63**:35-71.

McMahon, B.R. and Wilkens, J.L. (1983). Ventilation, perfusion, and oxygen uptake. In: Mantel, L.H. and Bliss, D.E. (eds.), *The Biology of Crustacea, Vol. 5. Internal Anatomy and Physiological Regulation.* New York: Academic Press, pp. 289-372.

McMahon, B.R., Sinclair, F., Hassall, C.D., DeFur, P.L. and Wilkes, P.R.H. (1978). Ventilation and control of acid-base status during temperature acclimation in the crab *Cancer magister*. *Journal of Comparative Physiology*, **128B**:109-116.

McMahon, B.R., Chu, K.H., Mak, E.M.T. (1995). Development of the heart in the shrimp *Metapenaeus ensis*. In: Lavens, P., Jaspers, E. and Roelants, I. (eds.) Larvi'95 - *Fish and Shellfish Larviculture Symposium*, European Aquaculture Society, Special Publication No. 24. Belgium: Gent, pp. 470-473

McMahon, B.R., Smith, P.G.S. and Wilkens, J.L. (1997). Invertebrate circulatory systems. In: Danzler, W.H. (ed.) *Handbook of Physiology*, Section 13, *Comparative Physiology. Vol. 2.* New York: Oxford University Press, pp. 67-88.

McMahon, B.R., Bourne, G.B. and Chu, K.H. (in press). Invertebrate cardiovascular development. In: Burggren, W.W. and Keller, B. (eds.) *Development of the Circulatory System: Molecules to Organisms*. New York: Cambridge University Press.

Panikkar, N.K. (1968). Osmotic behavior of shrimps and prawns in relation to their biology and culture. *FAO Fisheries Report*, **57**:527-538.

Parado-Estepa, P., Ferraris, R.P., Ladja, J.M., and DeJesus, J.G. (1987). Responses of intermolt *Penaeus indicus* to large fluctuations in environmental salinity. *Aquaculture*, **64**:175-184.

Phillips, J.E., Hanarhan, J., Chamberlin, M. and Thomson, B. (1986). Mechanisms and control of reabsorption in insect hindgut. *Advances in Insect Physiology*, **19**:329-422.

Potts, W.T.W. (1954). The energetics of osmotic regulation in brackish and fresh water animals. *Journal of Experimental Biology*, **31**:618-630.

Potts, W.T.W. and Parry, G. (1964). *Osmotic and Ionic Regulation in Animals.* Oxford: Pergamon Press, pp. 1-403.

Preston, N. (1985). The combined effects of temperature and salinity on hatching success and the survival, growth, and development of the larval stages of *Metapenaeus bennettae* (Racek and Dall). *Journal of Experimental Marine Biology and Ecology,* **85**:57-74.

Prosser, C.L. (1973). Water: Osmotic balance hormonal regulation; inorganic ions. In: Prosser, C.L. (ed.). *Comparative Animal Physiology*. Philadelphia: Saunders, pp. 1-110.

Prosser, C.L. (1991). Temperature. In: Prosser, C.L. (ed.). *Environmental and Metabolic Animal Physiology*. New York: Wiley Liss, pp. 109-165.

Rosas, C., Barrera, G. and Lazarc-Chavez, E. (1989), Effecto de las variaciones de la salinidad y de la temperatura estacional sobre el consumo de oxigeno de *Callinectes rathbunae*, *C. contreras y Callinectes similis* (Crustacea: Portunidae). *Tropical Ecology*, **30**:193-204.

Rudy, P.P. (1967). Water permeability in selected decapod Crustacea. *Comparative Biochemistry and Physiology*, **22**:581-589.

Sandoz, M. and Rogers, R. (1944). The effect of environmental factors on hatching, moulting and survival of zoea larvae of the blue crab *Callinectes sapidus* Rathburn. *Ecology*, **25**:216-228.

Schwartzkopff, J. (1955). Vergleichende Untersuchungen der Herzfrequenz bei Krebsen. *Biologisches Zentralblatt*, **74**: 480-497.

Shaw, J. (1961a). Studies on ionic regulation in *Carcinus maenas* (L.). I. Sodium balance. *Journal of Experimental Biology*, **38**:135-152.

Shaw, J. (1961b). Sodium balance in *Eriocheir sinensis* (M. Edw.). The adaptation of the Crustacea to fresh water. *Journal of Experimental Biology*, **38**:153-162.

Smith, R.I. (1967). Osmotic regulation and adaptive reduction of water permeability in a brackish-water crab, *Rhithropanopeus harrisi* (Brachyura, Xanthidae). *Biological Bulletin*, **133**:643-658.

Spaargaren, D.H. (1973). The effect of salinity and temperature on the heart rate of osmoregulating and osmoconforming shrimps. *Comparative Biochemistry and Physiology*, **45A**:773-786.

Spaargaren, D.H. (1974). A study on the adaptation of marine organisms to changing salinities with special reference to the shore crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology*, **47A**:499-512.

Spaargaren, D.H. and Achituv, Y. (1977). On the heart rate response to rapid temperature changes in various marine and brackish water crustaceans. *Netherlands Journal of Sea Research*, **11**:107-117.

Spicer, J.I. (1994). Ontogeny of cardiac function in the brine shrimp *Artemia franciscana* Kellogg 1906 (Branchiopoda: Anostraca). *Journal of Experimental Zoology*, **270**:508-516.

Spicer, J.I. and Morritt, D. (1996). Ontogenic changes in cardiac function on crustaceans. *Comparative Biochemistry and Physiology*, **114A**:81-89.

Stephens, P.J. and Atwood, H.L. (1982). Thermal acclimation in a crustacean neuromuscular system. *Journal of Experimental Biology*, **98**:39-47.

Taylor, A.C. (1977). The respiratory responses of *Carcinus maenas* (L.) to changes in environmental salinity. *Journal of Experimental Marine Biology and Ecology,* **29**:197-210.

Taylor, E.W. (1991). Kinetic studies on the association and dissociation of myosin subfragment 1 and actin. *Journal of Biological Chemistry,* **266**:294-302.

Taylor, E.W., Butler, P.J. and Al-Wassia, A. (1977). The effect of a decrease in salinity on respiration, osmoregulation and activity in the shore crab, *Carcinus maenas* (L.) at different acclimation temperatures. *Journal of Comparative Physiology*, **119**:155-170.

Ting, Y.Y. (1970). Study on the oxygen consumption of grass shrimp, *Penaeus monodon* and sand shrimp, *Metapenaeus monoceros*. *Bulletin of Taiwan Fisheries Research Institute*, **16**:111-118.

Truchot, J.P. (1978). Mechanisms of extrcellular acid-base regulation as temperature changes in decapod crustaceans. *Respiration Physiology*, **33**:161-176.

Varo, I., Taylor, A.C., Navarro, J.C. and Amat, F. (1991). The effect of temperature and oxygen tension on the rates of oxygen consumption of nauplii of different strains of *Artemia* acclimated to low salinity. *Marine Ecology Progress Series*, **76**:25-31.

Villarreal, H. (1990). Effects of temperature on oxygen consumption and heart rate of the Australian crayfish *Cherax tenuimanus* (Smith). *Comparative Biochemistry and Physiology*, **95A**:189-193.

Wickins, J.F. (1976). Prawn biology and culture. *Oceanography and Marine Biology*: *an Annual Review*, **14**:435-507.

Wilkens, J.L. and McMahon, B.R. (1992). Intrinsic properties and extrinsic neurohormonal control of crab cardiac hemodynamic. *Experientia*, **48**:827-833.

Wilkens, J.L. and Walker, T.L. (1992). Nervous control of crayfish cardiac hemodynamics. *Comparative Physiology*, **11**:115-122.

Williamson, D.I. (1982). Larval morphology and diversity. In: Abele, L.G. and Bliss, D.E. (eds.), *The Biology of Crustacea. Vol. 2. Embryology, Morphology, and Genetics*. New York: Academic Press, pp. 43-110.

Yamagishi, H. (1990). Physiological change of the heart beat in juvenile stage of the isopod crustacean, *Ligia exotica*. *Zoological Science*, **7**:1037.

Yamagishi, H. and Hirose, E. (1992). Nervous regulation of the myogenic heart in early juveniles of the isopod crustacean *Ligia exotica*. In: Hill, R.B., Kuwasawa, K., McMahon, B.R. and Kuramoto, T., (eds.), *Phylogenetic Models of Functional Coupling of the CNS and Cardiovascular System. Comparative Physiology*. Basel: Karger, pp. 141-148.

Zainal, K.A.Y., Taylor, A.C. and Atkinson, R.J.A. (1992). The effect of temperature and hypoxia on the respiratory physiology of the squat lobsters, *Munida rugosa* and *Munida sarsi* (Anomura, Galatheidae). *Comparative Biochemistry and Physiology*, **101A**:557-567.